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A CYTOCHROME FROM HALOTOLERANT BACTERIA[1]

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(Received for publication, July 15, 1953)

While various types of cytochrome have been reported to occur in widely different tissues and cells, only a few of their components have actually been obtained as soluble preparations. Especially, no successful isolation of soluble cytochromes from bacterial sources has so far been achieved. We have recently succeeded in obtaining a soluble cytochrome from strains of halotolerant bacteria. Furthermore, it has been suggested that this cytochrome may be of a novel type differing from any of the hitherto known ones. As a new type of cytochrome we designate it as cytochrome b_4, discriminating from b (b_1, b_2, b_3) and c.

The purpose of this paper is to describe the procedure of purification and some of the properties of cytochrome b_4.

EXPERIMENTAL

Bacterial Cultures—Two strains of halotolerant bacteria were used for the extraction of the cytochrome. The one was a motile, Gram-positive bacillius and the other a Gram-negative coccus.[1] It has later become likely by the experiments of one of us that the two strains may be two different forms of one and the same species of bacteria, for the two strains can be converted into each other under certain condition.[3]

Both strains are aerobic and exhibit an unusual tolerance toward extraordinarily high salt concentrations. Such a halotolerance of these organisms was of considerable advantage for the present purpose, since the cells of these bacteria grown on salt-rich

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1) A preliminary report of this work has already been presented (*1*).

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2) We are indebted to Mr. H. Yamada of the Research Laboratory of Japan Marine Products Co., Ltd. for fournishing these strains.

3) The mutual transformation of bacterial types observed by one (Mori) of us will be reported elsewhere.

media had been found to be readily ruptured by simple exposure to distilled water or other hypotonic aqueous solutions (*1*, *2*).

The cultivation of these bacteria was carried out at 35° for 40–50 hours on nutrient plates of the following compositions: peptone 1, meat extract 1, potassium nitrate 3, sodium chloride 10, agar 2.5 and tap water 100. The pH was adjusted to 6.8–7.0 with 1 *M* sodium hydroxide.

Extraction and Purification of Cytochrome—In extracting the cytochrome from the above two strains, we used essentially the same procedures. The preparations obtained from both strains were apparently identical. In the following, therefore, only the experiences with the latter strain will be described.

The cells harvested from the plates were washed several times with 10 per cent saline solution until no nitrite could be detected in the washings. The washed cells were then heavily suspended in distilled water and completely dried by freeze-drying. 10 g. of the lyophilized cell powder were dispersed in 200 ml. of *M*/50 phosphate buffer of pH 7.0 with the aid of a Potter-Elvehjem glass homogenizer. After keeping the suspension in a refrigerator for 14–20 hours, the ruptured cell debris were throughly removed by centrifugation and discarded. The slightly turbid reddish supernatant (160–170 ml.) containing the cytochrome was chilled to −2° by a freezing mixture consisting of cracked ice and common salt. 0.75 volume of cold acetone, cooled to −10°, was then added dropwise to the supernatant under vigorous stirring. The acetone concentration of the mixture became 43 per cent by volume in this step and the final temperature was about −5°. After standing for 10 minutes in the cold, the colorless precipitate was centrifuged off and discarded. The pH of the supernant was adjusted to 5.3 by the addition of a small amount of 1 *M* acetic acid. The cytochrome was then precipitated by a further addition of cold acetone (1.25 volumes of the original supernatant) to raise the acetone concentration to 66 per cent by volume; the temperature having to be kept below −5°. The reddish precipitate was collected by centrifugation and dissolved in 25 ml. of *M*/50 phosphate buffer of pH 7.0. The acetone fractionation was then repeated as before and the precipitate obtained between 43 and 66 per cent acetone concentrations was again taken into 25 ml. of the phosphate buffer. Then, the pH of the solution was decreased to 4.2 by a dropwise addition of 2 *M* acetic acid in the cold (−2°). The acidic proteins thus precipitated were rapidly removed by centrifugation and discarded. The supernatant was immediately neutralized to pH 7.0 with 1 *M* sodium hydroxide. A third acetone fractionation was then applied to the neutralized solution as before. The red precipitate thus obtained was dissolved in a minimal volume of distilled water and dried by freeze-drying. The yield of the dried preparation was 60–70 mg.

Properties of Dried Preparation—The dried preparation of crude cytochrome b_4 obtained by the above procedure was pink colored powder and completely soluble in water or phosphate buffer to give a clear red solution. The cytochrome was readily reduced by the addition of a small amount of either sodium hydrosulfite ($Na_2S_2O_4$) or sodium borohydride ($NaBH_4$). On reduction, the red color of the solution was intensified and became rather scarlet. The reduced solution was relatively unstable even at neutral reactions and the color slowly faded at room temperature. The oxidized cytochrome, however, appeared to be more stable in neutral media. Under acidic conditions it was rather labile at ordinary temperature.

Absorption Spectra—The novelty of the cytochrome was first recognized by qualitative observations of the intact cell suspensions with a hand spectroscope. This was further indicated by a quantitative determination of the light absorption of the extracted preparation using a Beckman Model DU spectrophotometer. Fig. 1 shows the spectrophotometric absorption curves for both the oxidized and reduced preparations. In the measurement of the reduced spectrum $NaBH_4$ was used as reducing agent rather than the usual $Na_2S_2O_4$, since the former having no absorption in the near ultraviolet region has been shown to be more suitable for this purpose. It was apparent, as will be seen in Fig. 1, that the preparation has absorption curves characteristic to a pigment of cytochrome nature. Three distinct peaks were observed in the spectrum after reduction, namely, α-band at 554 mμ, β-band at 521 mμ and the most intense γ- or Soret band at 418 mμ. In the oxidized spectrum the Soret band was shifted to 410 mμ and its height was somewhat lowered as compared with that of the reduced cytochrome. Furthermore, the sharp α- and β-bands of the reduced cytochrome disappeared on oxidation; instead, a diffuse absorption with a maximum at about 527 mμ and a hump at about 550 mμ became visible. A small peak at 360 mμ also appeared in the oxidized spectrum. The spectrum of the oxidized form may certainly depend much on hydrogen ion con-

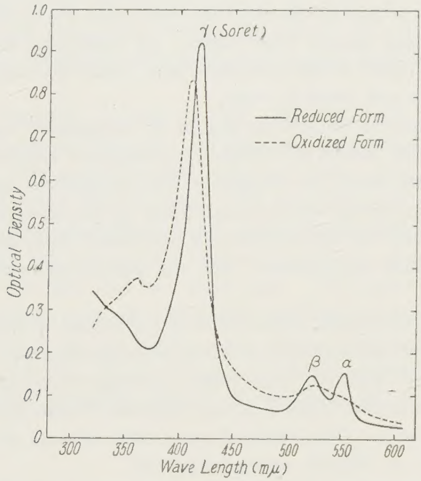

FIG. 1. Absorption spectra of the cytochrome preparation at pH 7. Eight milligrams of the preparation were dissolved in 4 ml. of $M/20$ phosphate buffer. Light path: 1 cm. The cytochrome was reduced by the addition of a small amount of $NaBH_4$.

centrations of the medium. Precise investigations upon this effect have not yet been carried out. These spectrophotometric features, obviously indicating the cytochrome nature of this pigment, were not identical with those of the hitherto known cytochromes.

Heme-group of Cytochrome b_4—The heme part of the cytochrome b_4 can be readily split from the protein part by the action of mineral acid.

The cytochrome was dissolved in 50 per cent acetone. Acidifying the solution by the addition of a small amount of acetic acid and on shaking with ether the red color can scarcely be transferred into the ether layer. On the contrary, by the action of stronger acids, *e.g.* hydrochloric acid, it was readily extracted with ether. The clear solution was then washed with water two or three times to remove acetone and the acid. The heme part was then drawn from the ether layer into a small volume of dilute sodium carbonate solution two or three times. By acidifying the combined extracts the hematin was precipitated in fine granular form, which was collected on centrifuge, washed well with water and dried *in vacuo.*

Preliminary attempts to obtain the hemin in crystallized from (Teichmann's test) did not succeed. By means of a mixture of methanol and hydrogen chloride the formation of methylester could be attained. The methylester had lost solubility in slightly alkaline aqueous media, without any change in the spectrum of pyridine-heme. From the solution in ether by the addition of petroleum ether one obtained granular deposit of microcrystalline appearance. For the lack of material this attempt was pursued no further.

The hemin as well as original cytochrome b_4 are soluble in slightly alkaline media, but neither of them shows any solubility in dilute acids, in contrast to that of cytochrome *c*. The hematin is hardly reduced, if any hemochromogen-forming base is absent, while it gives intense pink colour of pyridine-heme on addition of some pyridine with $Na_2S_2O_4$. The main absorption bands of the reduced form have maxima at 554 and 523 mμ in accordance with those of original cytochrome itself.

The position of the maxima suggests that the hemin would have an intermediate structural form between protohemin and cytochrome *c*. In fact, it was found that cytochrome b_4 can be readily transformed either into cytochrome *c* or into *b* within the bacterial cell under suitable conditions. Although results of precise investigations upon transformations of cytochromes will be reported elsewhere, some informations will be given below for confirming the assumption of the structure of cytochrome b_4.

1. Proto- and Hematoreaction: One of the present authors (Mori) has investigated upon the transition and establishment of equilibrium between proto- and hematohemin in pyridine-hydrochloric acid mixtures of various proportions and dilutions.[4] With the aid of graphical representations one can determine the most favorable conditions for the formation of either proto- or hematohemin, and it was found that the former is realized in a medium of pyridine and 30 per cent HCl in the ratio of 1:2, and the latter in that in the ratio of 1:20, respectively.

The hemin from cytochrome b_4 was treated in these two kinds of media separately, under short heating (15 minutes) on a water bath. After cooling and diluting the colourless mother liquors were filtered off. The precipitates were washed well, dissolved in pyridine containing dilute soda solution. On reduction by $Na_2S_2O_4$ the absorption spectra of the resulted pyridine-hemes were as follow.

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[4] Unpublished.

In the protoreaction	558,	527 mμ
In the hematoreaction	549,	520 mμ

Both the transition reactions of b_4-hemin are thus clearly shown.

These results further suggest the existence of some loosely bound hapt-group at the vinyl-side chains of prothemin.[5]

2. *Sulfur Test:* Theorell (*3*) has reported a proof of a sulfur-bridge at the side chain of hemin-group in the cytochrome *c*-molecule. We had attempted a preliminary test on the presence of sulfur atoms in the hapt-group of b_4-hemin. From the above mentioned result it is certain that the hapt-group can be easily split off by the action of a stronger acid.

b_4-hemin was, therefore, at first treated with nitric acid on a water bath. The hemin part was decomposed strongly and dissolved in acidified water and filtered off. This fraction gave, after total digestion, only very little amount of $BaSO_4$ by the addition of $BaCl_2$ in a dilute HCl solution. The insoluble faint yellow precipitate of the nitric acid decomposition was collected and completely digested with the aid of a mixture of hydrogen peroxide and concentrated aqueous ammonia two times and a mixture of potassium chlorate and nitric acid on a water bath. The aqueous solution of the digested material gave remarkable precipitate of $BaSO_4$ with $BaCl_2$ solution. Though the preparation of the hemin was not yet highly pure, we may assume the presence of a sulfur containing group in the side chain of hemin b_4-molecule with large possibility and this fact may suggest some genetical relation of cytochrome b_4 to *c*.

DISCUSSION

The cytochrome preparation reported in this paper, though crude as yet, seems to have important bearings on the knowledge of cytochrome chemistry in several respects. First, it is the first soluble cytochrome obtained from bacterial cells. The cell-free preparations of bacterial cytochromes have already been reported, but they were always associated with cellular particles, which prevented further purification. Judged from its properties, the preparation reported here appears to be completely soluble and free from cells tructures. This preparation, therefore, would be of promise for further purification and for precise investigation of the chemical nature of the bacterial cyto-

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5) These reactions are totally absent in cytochrome *c*. The side linkages of the heme part of cytochrome *c* are so steadfast that they can not be affected by such mild treatments.

chrome. Although the cytochrome is not yet obtained as pure preparation, the absorption spectra presented in this paper strongly support the view that it is a new type of cytochrome. For comparison, the absorption bands of some of the typical red cytochromes are presented in Table I together with those of cytochrome b_4.

TABLE I

Absorption Bands of Reduced Cytochromes

Cytochrome	Position of band (mμ)			Reference
	α	β	γ (Soret)	
b_4 (halotolent bact).	554	521	418	—
b (heart muscle)	564	534	432	(*4*)
b_1 (*Escherichia coli*)	560	530	?	(*5*)
b (yeast)	562	532	?	(*6*)
b_2 (yeast)	556.5	530	420	(*7*)
b_3 (green leaves)	558.6	529.3		(*10*)
c (heart muscle)	550	521	415	(*4*)
c_1 (heart muscle)	552	522	416	(*8*)
f (green leaves)	555	520	420	(*9*)

Of the cytochromes that have ever been described, c_1, b_2, and f in Table I resemble to b_4. Cytochromes c_1 and b_2 are, however, distinguished decisively from b_4 in their spectra of the reduced form in the presence of pyridine (α-band of pyridine-c_1 is placed at 550 mμ and that of pyridine-b_2 at the coincident position with that of pyridine-protoheme). The closest resemblance in the absorption spectra can be found between b_4 and f. There might exist some relations between them, though the properties of the bacterial cytochrome, so far investigated, differ markedly from those of the chloroplast pigment. The α-band of b_4 is not so sharp as that described for f. The normal potential of the former is admittedly even lower than that of cytochrome c. It appears to lie approximately in the range between that of b and c. Finally the solubility of b_4 in ammonium sulfate solutions seems to be far larger than that of f.

At the end of this discussion, it may be noted that this cytochrome was found in halotolerant bacteria, whose metabolism might involve some unusual mechanisms responsible for their tolerance toward environments with high osmotic pressure. Further investigations have, however, shown, that the cytochrome type is maintained unchanged in the bacterial cells cultured on common broth agar plates after some trainings. The apparently same cytochromes are also found to be present in other various kinds of organisms under certain conditions.[3)] In these respects we came to a conception that the cytochrome of b_4-type may have more general meanings in the cellular respriation.

SUMMARY

A procedure was described for the extraction and purification of a soluble cytochrome from strains of halotolerant bacteria. The absorption spectra of this preparation were measured in the visible and near ultraviolet region. The hemin group of the cytochrome was isolated and some of its properties were investigated. From these results it was suggested that this cytochrome may be a hitherto unknown one. Relations to other kinds of cytochrome were also discussed to a certain extent.

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ON THE KINETICS OF THE HUMAN BLOOD CHOLINESTERASE

III. INHIBITION OF CHOLINESTERASE BY URETHANE

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(Received for publication, July 20, 1953)

Based on the results of kinetic studies reported in previous papers (*1*), it was shown that the hydrolysis of acetylcholine by cholinesterase, its substrate inhibition and the inhibition by eserine can be expressed by the following equations, where E,S,P, and G are cholinesterase, acetylcholine, final reaction product, and eserine, respectively.

$$E+S \rightleftharpoons ES \qquad (1)$$
$$ES \longrightarrow E+P \qquad (2)$$
$$ES+S \rightleftharpoons ESS \qquad (3)$$
$$E+G \rightleftharpoons EG \qquad (4)$$

Interestingly enough, the entropy change in the reaction of ES formation was found to be a positive quantity. The fact seems to be of great importance in the consideration of the interrelation between the physical state of the protein part and the function of the enzyme (*2*).

Present communication deals with the effect of urethane on human blood cholinesterase. Heim and Fahr (*3*) reported that cholinesterase is inhibited by urethane in its higher concentrations, but is activated by it in its lower concentrations. Present investigation has proved that the inhibition by urethane is reversible in its lower levels, while it is irreversible in its higher concentrations. Similar phenomena have recently been reported concerning cholinesterase and $HgCl_2$ by Goldstein (*4*) and concerning invertase and urethane by Johnson *et al.* (*5*).

The activating effect of urethane, which was stated by Heim and Fahr, could not be observed in the concentrations of urethane applied in the present experiment.

EXPERIMENTAL

Activity measurement of cholinesterase preparation was carried out by the manometric method described by Ammon (*6*). The preparation used was red cell suspension described in the previous communication. pH of the reaction medium was held at

7.4 throughout the whole experiments. Temperature was held at 37.5° except in the experiment of temperature change. For the determination of urethane concentration, N content was estimated by microkjeldahl method every time. Urethane solution was added either to the enzyme solution (side arm) or to the substrate solution (main vessel). In the former, except in experiments with varied incubation time, the enzyme solution was first incubated with urethane for 10 minutes at room temperature and then for 10 minutes at 37.5°. The activity was expressed in terms of cmm CO_2 evolved every 10 minutes.

RESULT AND DISCUSSION

Reversibility of the Urethane Inhibition—Changes in the activity of cholinesterase were measured every 10 minutes with 0.0025 *M* acetycholine solution containing urethane in varied concentrations. Fig. 1 (II–VI) shows a plot of enzyme activity thus measured against time. The activity of enzyme decreases gradually with increasing urethane concentration. The reaction proceeded linearly in each urethane concentration. Now, enzyme was first incubated with urethane of

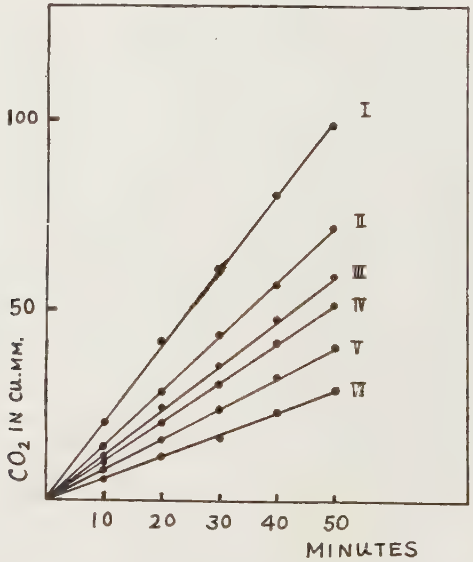

FIG. 1. Inhibition of cholinesterase by urethane, expressed by activity-time relationship. Final molarity of urethane was as follows: I, 0; II, 0.11; III, 0.16; IV, 0.21; V, 0.31 and VI, 0.41.

varied concentrations as indicated in Fig. 1 (II–VI) and then substrate solution was added, so that the final urethane concentrations were lowered to 1/26 of the initial concentrations. The activity was then found to be restored practically to the level of the control value (Fig. 1, I). The observation indicates that the inhibition of red cell cholinesterase by urethane in the above-mentioned concentrations is reversible. The equilibrium of the inhibition reaction seems to be established in relatively short time, since the reaction proceeded linearly from the starting point.

The Effect of Substrate Concentrations upon the Inhibition—Keeping the urethane concentration constant (0.21 *M*) and making that of the substrate variable, a pS-activity curve was obtained as shown in Fig. 2, II. The maximum of the pS-activity curve shifted to the right in the presence of urethane, as compared with that in the absence of urethane. A similar shift of pS-activity curve was reported by Augustinsson and Nachmansohn (*7*) in the reaction of eserine inhibition. Now, inhibition H can be expressed by

$$H=1-v_g/v_o \qquad (5)$$

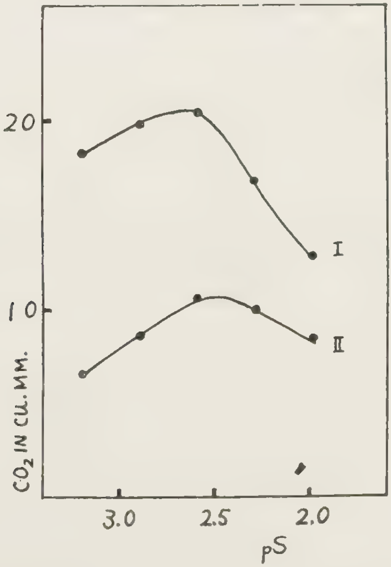

FIG. 2. pS-activity curves. Curve I, control; Curve II, with urethane (final concentration: 0.21 *M*).

where v_0 means the reaction velocity without urethane and v_g means that with urethane. The inhibition H by urethane (0.21 *M*) with varied substrate concentrations is given in Table I. For comparison, H by eserine (10^{-7} *M*) is also given in the Table I.

TABLE I

The Relationship between the Inhibition H and Substrate Concentrations

Substrate (*moles/liter*)	H	
	Urethane (0.21 *M*)	Eserine (10^{-7} *M*)
0.01	0.34	0.19
0.005	0.41	0.37
0.0025	0.47	0.48
0.00125	0.57	——
0.001	——	0.69
0.000625	0.63	——

The inhibition H increased as the substrate concentration decreased. It seemed thus likely that urethane reacts with the enzyme in competition with the substrate. The relative increase of H caused by the decrease of substrate concentration is, however, much smaller in urethane inhibition as compared with that in eserine inhibition, and the effect of the latter has been assumed as a true competition. Further and more detailed studies on the nature of the urethane inhibition should be required.

Relationship between the Enzyme Activity and the Urethane Concentration —

Fig. 3 is a plot of relative activities of the enzyme (taken from the data of the initial velocities given in Fig. 1) against the urethane concentrations. The decrease of activity in the Fig. 3 shows an exponential curve.

Fig. 4 is a plot of H against logarithm of the urethane concentration (G). Now, inhibition H is generally expressed by

$$H=\frac{G^n}{\phi^n+G^n} \tag{6}$$

where G means the concentration of the inhibitor. The curve in Fig. 4 agrees with that theoretically obtained from Eq. 6 when n=1. Thus the number of urethane molecule reacting to one enzyme molecule may be assumed to be approximately one. Further studies on this point will be reserved for the next communication.

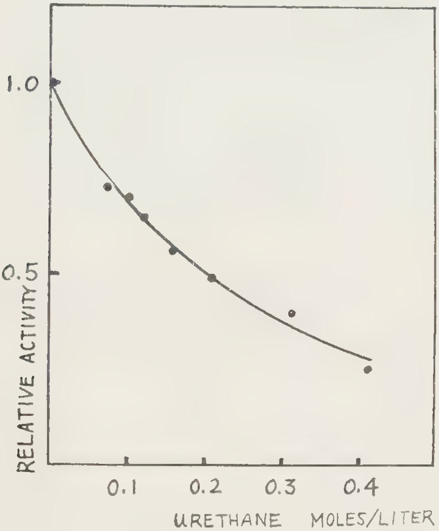

FIG. 3. Relationship between the cholinesterase activity and urethane concentrations. Ordinate, relative activity; abscissa, concentration of urethane.

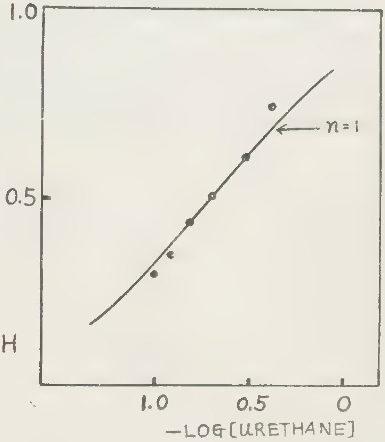

FIG. 4. The fit of the calculated sigmoid curve of first order to experimental data. Ordinate, the degree of inhibition; abscissa, logarithm of urethane concentrations.

The Inhibition by Urethane in Its Higher Concentrations—All the results dercribed so far were obtained by the determination of remaining activities of enzyme affected by urethane which was incubated simultaneously with substrate. It was further proved that when the enzyme was previously incubated with urethane, namely 10 minutes at room temperature and 10 minutes more in thermostat, no inhibition at all was brought about by urethane in its concentrations applied in Fig. 1. Further increase in urethane concentration, however, caused inhibition under the same condition. The remaining activities thus estimated are shown in Fig. 5, in which the final concentration of urethane was 1/26 of that of initial incubation. It has been proved in Fig. 1 that urethane caused no inhibition when enzyme was previously incubated with urethane in these final concentrations. As indicated in Fig. 5 II–V, the remaining activity decreased with increase of inbubated urethane concentration and the hydrolysis by remaining activity proceeded linearly. All the results thus obtained indicate that the inactiviation by urethane in its higher level is an irreversible reaction. It could not be reactivated by incubation, *i. e.* by dilution with substrate solution. In the present stage of investigation, however, the apparent linear relationship between remaining activities and time (Fig. 5) may be explained, when we consider the results of later reactions, by the existence of both the reversible and the irreversible reactions. The relation between the remaining relative activity and urethane concentration is shown in Fig. 6; the curve is wholly different from that given in Fig. 3.

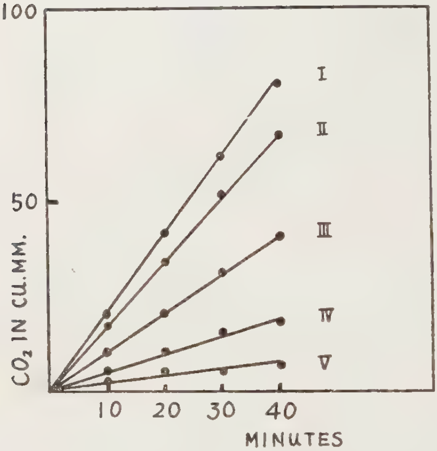

FIG. 5. Relationship between time and the remaining enzyme activity after incubation with urethane. Molarity of urethane during the incubation was as follows: I, 0; II, 0.72; III, 0.80; IV, 0.83; V, 0.90. Final concentrations of urethane were 1/26 of the above mentioned values.

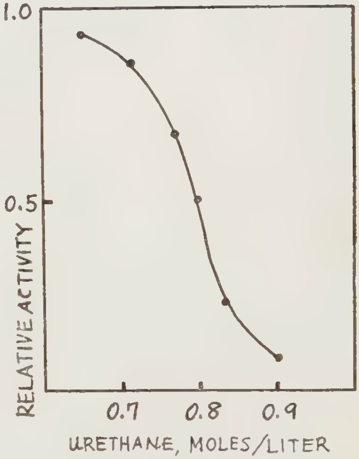

FIG. 6. Inhibition by urethane in its higher concentrations. Ordinate, remaining enzyme activity; abscissa, concentration in moles urethane.

Inhibition-pG curve obtained from Fig. 6 is shown in Fig. 7. The curve below H=0.5 fits the theoretical curve when n is taken as about 10. For the H-value above 0.5, however, higher value of n must be considered. In these points, the present reaction is evidently different from that shown in Fig. 4, indicating that the mode of inhibition by urethane in its higher levels is different from that in lower ones. The sigmoid H-pG curve (Fig. 7), its deviation from the theoretical curve above H=0.5, and the results given in Fig. 5, all indicate that a irreversible reaction predominates in the inhibition by urethane in the higher concentration range investigated.

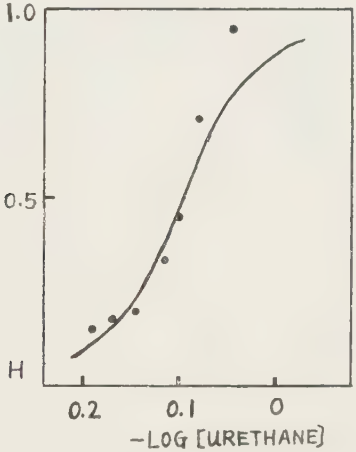

FIG. 7. Relationship between the inhibition by urethane in its higher concentrations and the logarithms of urethane concentrations

Effects of Substrate Concentrations—It is shown in Table II that the inhibition H is maximum at the substrate concentration of 0.0025 *M* (this is the optimal concentration for the erythrocyte cholinesterase) and it decreases on both sides of this substrate concentration. This seems to be of some interest in connection with the observation made by Goldstein (*8*), in which he has proved that cholinesterase is more labile against heat at its optimum pH.

Incubation Time—Remaining activities were determined after incubation with urethane lasting varied length of time, urethane concentration being kept constant. The activities determined are shown in Fig. 8 (full line).

The activity decreases exponentially with increasing incubation time. Relation between the logarithm of remaining activity and incubation time could be expressed by a straight line given in Fig. 8 (dotted line). From this it will be assumed that the inactivation of

TABLE II

The Relationship between the Inhibition by Urethane in its Higher Concentrations and the Substrate Concentrations

(The values of H given in the table are obtained by 0.797 *M* urethane)

Substrate (*moles/liter*)	H
0.01	0.27
0.005	0.32
0.0025	0.47
0.00125	0.34
0.000625	0.29

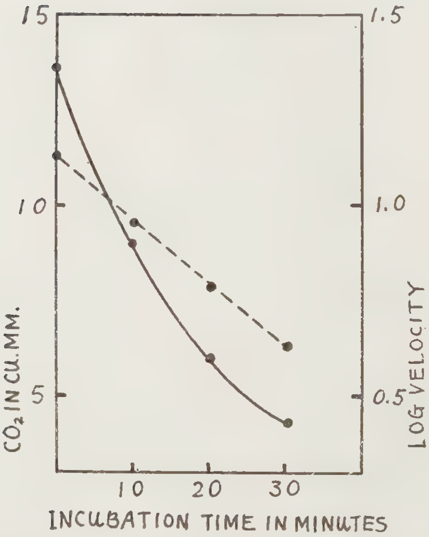

FIG. 8. Relationship between remaining activity and incubation time in the inhibition by urethane in its higher concentrations. Ordinate (left), remaining activity in CO_2 cu.mm.; ordinate (right), logarithm of remaining activity, abscissa, incubation time in minutes.

enzyme by urethane, in the above mentioned concentration, proceedes according to first order kinetics.

Effect of Temperature upon the Inhibition Reactions of Both Types—From the results described above, it was concluded that there are two different modes of inhibition by urethane according to its concentration. Now, the temperature effects upon inhibition H of both types of the reactions were studied. The result shown in Table III indicates that the temperature effect is small in the inhibition by urethane in lower concentrations. The inhibition increases with decrease of temperature.

TABLE III

Substrate concentration: 0.0025 *M*
Hl: inhibition by 0.207 *M* urethane
Hh: inhibition by 0.79 *M* urethane
He: inhibition by 10^{-7} *M* eserine

	Urethane		Eserine
t°C	Hl	Hh	He
15	——	——	0.27
20	0.62	0	0.30
25	——	——	0.34
26	0.58	0	——
30	——	——	0.38
31	0.54	0	——
35	——	0.12	——
37.5	0.47	0.45	0.48
39	——	0.62	——

In higher urethane concentrations, however, more pronounced temperature effect was observed in the inhibition at temperatures above 35°. At the temperature below 30°, no inhibition occurred with urethane of 0.797 *M* concentration. Each of these two types of urethane inhibition is thus proved to be different in its reaction nature. It may be pointed out further that in the temperature effect of urethane inhibition in its lower concentrations, contrary to that of eserine, H becomes greater as the temperature decreases (Table III).

Since the inhibition H by urethane in its higher concentrations is expressed by the remaining activity of enzyme determined after incubation with urethane in certain length of time, H may be regarded as a value corresponding to the inactivation of the enzyme. The plot of logarithm of H against 1/T gives a straight line as shown in Fig. 9. The Arrhenius energy calculated from the slope of the line is about 65 kcal. This is approximately of the same order of magnitude as the activation

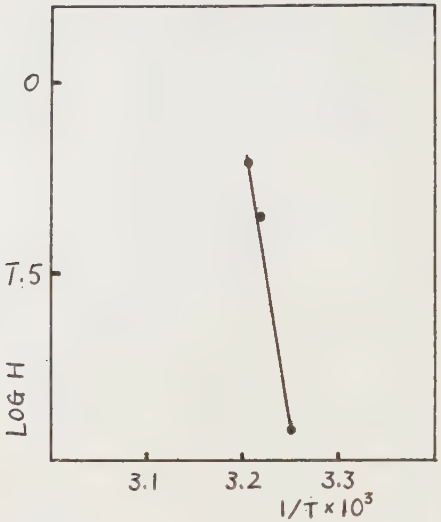

FIG. 9. Change of the logarithms of H with the reciprocal of absolute temperature in the inhibition by urethane in its higher concentrations.

energy observed in the denaturation reaction of most of the proteins. From this value and the results obtained in Figs. 5 and 8, it may be inferred that the inhibition of cholinesterase by urethane in its higher levels is brought about through denaturation of enzyme protein or some similar reaction.

The present investigation was supported by Prof. Dr. K. Kaziro, to whom the author's deep gratitude is due.

SUMMARY

1. Urethane inhibits the cholinesterase in two qualitatively different ways as the incubated concentration of the former differs.

2. The inhibition of the enzyme by urethane in concentrations below 0.5 *M* is reversible; it increases with decreasing temperature.

3. When urethane is in concentrations above 0.5 *M* the inactivation becomes irreversible and it proceeds according to the first order reaction. It is of high order reaction in respect to the urethane concentration. Activation energy of the reaction was calculated to be 65 kcal.

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The Journal of Biochemistry, Vol. 40, No. 6, 1953

STUDIES ON AMINE DEHYDROGENASES.

I. BACTERIAL HISTAMINE- AND PUTRESCINE-DEHYDROGENASES

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As shown in the previous paper from this laboratory (*1, 2*), resting cells of various bacteria were able by their adaptive enzyme systems to oxidize many primary amines and the initial oxidation of the amines to aldehyde, hydrogen peroxide and ammonia was catalysed by an enzyme similar to animal diamine oxidase, though the bacterial enzyme seemed to be strictly specific for each amine, differing from the animal one which has been assumed to act on histamine and diamines widely (*3*). Recently, the bacterial enzymes were extracted from the cells of *Achromobacter sp.*, previously adapted to amines and the properties of histamine- and putrescine-destroying enzymes have been investigated in more detail, which are described in the present paper.

The bacterial enzymes were found to be dehydrogenases and to undergo coupling with oxygen molecule, not directly but via the respitatory systems. This fact differs from that observed in animal and plant amine oxidases, which have been reported as being able to react directly with molecular oxygen (*4, 5*). The dehydrogenases seemed to be flavo-protein, having flavin-adenine-dinucleotide as their prosthetic group, which was rather resistant against dialysis. The inhibition caused by methylene blue, Atabarine, and quinine were concluded to be due to the competition with the prosthetic group for their apoenzymes.

EXPERIMENTAL

Bacteria and the Culture—Achromobacter sp., used in this study, was the same strain as had been used in the previous paper (*1*). The cells from the 18-hours broth culture at 30°, were washed twice with water and used as the intact cells unadapted to amines. They were resuspended in *M*/50 phosphate buffer (pH 7.4), containing one mg. of amine dihydrochloride per ml. and shaken vigorously in air for three hours at 30°. The washed cells were taken as the intact cells adapted to the given amine. As the amine dehydrogenase was an adaptive enzyme and the activity in the intact cells decreased readily in the absence of the substrate (*1*), the washed cells should be used as rapidly as possible for the measurement of the activity and for the preparation of enzymes.

Enzyme Preparation from Bacteria—The washed cells were removed from the washings as well as possible, chilled to −5° and introduced into 100 volumes (vol./wet weight) of well-chilled acetone. The resulting flocky precipitate was rapidly filtered from acetone and immediately dried in vacuum, in a usual manner (*6*). The acetone-dried cells maintained the activity of amine dehydrogenase for one week in an evacuated desiccator (over calcium chloride) below 5°. By these acetone treatments, the respitatory enzyme system which is responsible for the coupling between amine dehydrogenase and molecular oxygen in the intact cells, was destroyed. The finely powdered acetone-dried cells were suspended in 200 volumes of water (*vol./wt.*) with continuous stirring. The viscous and opaque suspension, after standing for one hour in cold to complete the extraction of amine dehydrogenase, was centrifuged under 15,000 × *g*. for 20 min., to separate some insoluble particles. The clear supernatant was taken as the cell-free amine dehydrogenase preparation. Dialysis was carried out in a cellophane bag against running water. The amount of enzyme preparations is always expressed in mg. of the original acetone-dried cells.

Measurement af Activity—Oxygen uptake due to the oxidative destruction of the substrate at 30° in air, was measured by the conventional Warburg manometric technique (*2*, *6*) and the values of oxygen uptake stated below, were those calculated by subtracting auto-respiration from the experimental readings.

Activity of dehydrogenase was measured in evacuated Thunberg tubes by the reduction of 2,6-dichlorophenol-indophenol (*M*/5000) at 30°. All the data given below are the mean values obtained in duplicated experiments.

Materials—All the substrates used in this study were the same as those used in the previous work (*2*). Flavin-adenine-dinucleotide (purity, 60 per cent), riboflavin-5-phosphate, and Atabrine (2-methoxy-6-chloro-9-α-diethylamino-δ-pentylaminoacridine dihydrochloride) were kindly offered by Mr. T. Seki, Prof. K. Miyaki, and Prof. T. Yanagita, respectively. The concentrations of substrates described in the following are all final values in reaction mixtures.

RESULTS

Dehydrogenation of Histamine and Putrescine by Bacterial Enzymes—Table I gives the results of experiments, showing the effect of histamine and putrescine on the reduction of *M*/5000 2,6-dichlorophenol-indophenol by various intact cells, acetone-dried cells, or cell-free extracts of *Achromobacter sp*. The reduction was accelerated only by the coexistence of amine and the cells previously adapted to the amine, or their preparations, indicating that histamine and putrescine are dehydrogenated by bacterial adaptive enzymes and the dehydrogenation are specific for each amine.

The amine-amine dehydrogenase systems did not accelerate, however, the reduction of methylene blue ($M/5000$) even in the presence of riboflavin-5-phosphate ($M/1000$) as shown in Table II.

TABLE I

Reduction of $M/5000$ *2,6-Dichlorophenol-indophenol by Amine-amine Dehydrogenase System*

(At 30°, in $M/50$ phosphate buffer, pH 7.4, total volume 2.5 ml.)

Enzyme	Discoloration time		
	Control	Histamine $M/1000$	Putrescine $M/1000$
	min.	*min.*	*min.*
Bacteria unadapted to amines.			
Intact cells, 1 mg.	53	55	48
Acetone-dried cells, 3 mg.	100<	100<	100<
Bacteria adapted to histamine.			
Intact cells, 1 mg.	35	15	—
Acetone-dried cells, 3 mg.	100<	7.5	100<
Cell-free extract, 5 mg.*	85	13	—
Bacteria adapted to putrescine.			
Intact cells, 1 mg.	38	40	13
Acetone-dried cells, 3 mg.	100<	100<	20
Cell-free extract, 5 mg.*	100<	100<	21

* Expressed in the amount of the original acetone-dried cells.

As shown in Table I, highly active bacterial amine dehydrogenases were extracted from the cells in a good yield, by the method described in the experimental part. Thus the putrescine-destroying activity per weight of the acetone-dried bacterial cells (previously adapted to the amine) was more than 100 times of that of the acetone-dried hog kidney, under their respective optimal conditions (*3*). As to the activity per mg. of protein, the cell-free extract of the bacteria corresponded to more than 50 times of the activity of the so-called ' partialy purified animal diamine oxidase preparation ' (*4*) (the supernatant of the heat-treated extract of the latter powder).

Co-enzyme of Histamine- and Putrescine-dehydrogenases—Both amine dehydrogenases were rather resistant to the dialysis. Some decrease of the activity, which was observed after 24 to 48 hours' dialysis, could be recovered by the addition of a minute amount of flavin-adenine-dinucleotide (FAD). Riboflavin and its 5-phosphate, however, were scarcely effective for the recovery. These results, summarized in Table

TABLE II

Reduction of M/5000 Methylene Blue

(Under the same conditions as in Table I)

Acetone-dried cells of *Achromobacter sp.* previously adapted to†	Discoloration time			
	Nil		*M*/1000 Flavin-5-phosphate	
	Control	*M*/1000 Substrate*	Control	*M*/1000 Substrate*
	min.	*min.*	*min.*	*min.*
Ethanolamine	100<	100<	87	12
Choline	100<	100<	92	8.5
Histamine	100<	100<	100<	100<
Putrescine	100<	100<	100<	100<

* The same one as used in the enzyme adaptation.

† The similar results were obtained by the use of the intact cells, though all discoloration times were shorter relatively.

TABLE III

Effect of Dialysis and FAD on Histamine- and Putrescine-dehydrogenases

Enzyme*	Discoloration time	
	Control	With *M*/1000 substrate
	min.	*min.*
Histamine dehydrogenase		
Original cell-free extract	100<	7.5
8-hours dialysate	100<	9.5
24-hours dialysate	82	26
„ „ +20γ FAD	78	6.0
„ „ +*M*/1000 riboflavin	80	23
„ „ +*M*/1000 „ -5-phosphate	67	20
Putrescine dehydrogenase		
Original cell-free extract	100<	20
48-hours dialysate	76	42
„ „ +20γ FAD	70	19
„ „ +*M*/1000 riboflavin	59	34

* Amount of enzyme tested corresponded to 5 mg. of the acetone-dried cells and the experimental conditions were the same as described in Table I.

III, might be considered as indicating that bacterial histamine- and putrescine-dehydrogenases are like the animal diamine oxidase reported by Zeller (*7*) and Kappler-Adler (*8*).

Inhibition by Methylene Blue, Atabrine, and Quinine—Table IV shows the effect of riboflavin, its 5-phosphate, methylene blue, quinine and Atabrine on the rate of destruction of histamine, and Table V gives the results of similar experiments on the destruction of putrescine. It was seen that the marked inhibition by methylene blue, quinine, and Atabrine (*M*/4000-*M*/1000) was prevented competitively in the presence of riboflavin and its 5-phosphate, and twice the concentration of riboflavin-5-phosphate was sufficient to give up these inhibition. These results indicate that methylene blue, Atabrine, and quinine, having somewhat similar structure to flavin as described by Rydon (*9*), act antagonistically with flavin and the 5-phosphate on the amine dehydrogenases within the concentration tested. The inhibition caused by methylene blue and Atabrine in higher concentration than *M*/1000 could, however, be prevented only partially by the presence of riboflavin-5-phosphate in correspondingly higher concentrations.

Fig. 1 shows the effect of early and late additions of riboflavin-5-phosphate and methylene blue on the oxidation of putrescine. It was seen from these results, that the previous presence of riboflavin-5-phos-

TABLE IV

Oxidation and Dehydrogenation of Hitamine in the Presence of Various Compounds

Inhibitor \ Antagonist of inhibitor	Nil	Riboflavin *M*/2000	Rivoflavin-5-phosphate	
			M/2000	*M*/100
	per cent	*per cent*	*per cent*	*per cent*
Nil	100	115	(105)	119
Quinine *M*/4000	65	112	100	120
„ *M*/3000	(40)	(90)	(70)	(120)
„ *M*/2000	8	35	27	65
Atabrine *M*/4000	(40)	—	(90)	(110)
„ *M*/3000	7	41	36	69
Methylene blue *M*/4000	55	77	81	—
„ „ *M*/3000	41	—	—	84
„ „ *M*/2000	2	17	—	—

All figures represent the relative activity at 30° in *M*/50 phosphate buffer (pH 7.4), taking the control in the absence of inhibitor and the antagonist as 100. Experiments were performed with intact cells previously adapted to histamine, except for dehydrogenation tests in which acetone-dried cells were used. The results obtained with the latter are given in parentheses.

TABLE V

Antagonism of Methylene Blue and Quinine with Riboflavin-5-phosphate in the Bacterial Destruction of Putrescine

Inhibitor \ Antagonist of inhibitor	Riboflavin-5-phosphate			
	0	$M/2000$	$M/1000$	$M/500$
	per cent	*per cent*	*per cent*	*per cent*
Nil	100	102	108	120
Methylene blue $M/4000$	57	79	90	108
" " $M/3000$	43	69	92	—
" " $M/2000$	5	25	65	94
Quinine $M/3000$	(40)	(90)	(110)	—
" $M/2000$	(10)	—	(60)	(100)

All figures represent the relative activity of putrescine-oxidation (in the case of methylene blue addition) and -dehydrogenation (in the case of quinine), taking the control as 100, under the same conditions as in Table IV, except the use of the cells adapted to putrescine.

phate was effective in preventing the inhibition caused by methylene blue, while the later addition of the phosphate was unable to reactivate the enzyme already inactivated by methylene blue.

DISCUSSION

From the experimental results described above, it may be concluded that bacterial histamine- and putrescine-dehydrogenases seem to be different adaptive enzymes but both a flavo-protein, having FAD as their prosthetic groups, which are rather resistant to dialysis in both cases. The addition of methylene blue, quinine, and Atabrine which have similar structure to that of flavin (*9*), however, seems to cause the displacement of the prosthetic group (FAD) and to result in the inactivation of the dehydrogenases. Riboflavin and its 5-phosphate, though having scarcely any activity as the prosthetic group, seem to compete with these flavin-antagonists (inhibitor) on the dehydrogenases and to prevent such displacement of FAD and the inactivation to be caused by them, within the concentrations tested here ($M/2000$–$M/4000$). While the dehydrogenases previously inactivated by these inhibitors were unable to recover their activity by the later addition of riboflavin-5-phosphate, the inactivation of the dehydrogenases by the addition of the flavin-antagonists was prevented competitively by the previous

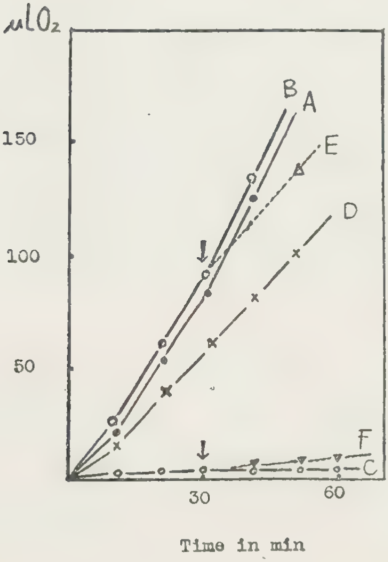

FIG. 1. Oxidation of putrescine by *Achromobacter sp.*, previously adapted to the amine.

At 30° in $M/50$ phosphate buffer (pH 7.4), in the presence of (B) $M/1000$ riboflavin-5-phosphate (RFP), (C) $M/2000$ methylene blue (MB), and (D) $M/1000$ RFP+$M/2000$ MB. (A) Control without these substances. Previous addition of (E) $M/1000$ RFP then $M/2000$ MB, and (F) $M/2000$ MB then $M/1000$ RFP at the time shown by an arrow.

presence of riboflavin-5-phosphate. The somewhat similar phenomenon concerning the enzyme-inhibition was recently reported by Suda *et al.* (*10*) on pyrocatecase, having ferrous ion as the coenzyme. In their case, the previous addition of *αα'*-bipyridyl as the inhibitor to the enzyme inactivated by dialysis, inhibited the recovery of the activity to be caused by addition of ferrous ion, while the already recovered activity of the dialysate by addition of ferrous ion was not inhibited by the latter addition of *αα'*-bipyridyl. Riboflavin-5-phosphate, though not the true prosthetic group of our bacterial amine dehydrogenases and the flavin-antagonists, seem to correspond to the ferrous ion and to *αα'*-bipyridyl respectively.

The antagonism of Atabrine, quinine, and methylene blue against flavins have already been reported by many workers on other cases

(*9*), and the inhibition of amine oxidases by methylene blue is a well-known fact (*2, 3*). Some years ago, Ota (*11*) found during his extensive studies on animal amine oxidase, that thionine and methylene blue inhibited the enzyme irreversibly differing from the inhibitor to compete with the substrate and concluded that these inhibitiors acted on the active center of the enzyme. However, our elucidation of the inhibition of bacterial amine dehydrogenases as being due to such antagonism with the prosthetic group, FAD, seems to be the first one, as far as we are aware. The inhibition of animal amine oxidase might be also due to the similar antagonism against FAD, the most probable prosthetic group (*12*).

Though the inhibition of bacterial amine oxidation by methylene blue (*M*/2000) was prevented competitively by the previous presence of riboflavin-5-phosphate (*M*/1000) (in the case of intact cells), the amine-amine dehydrogenase system did not accelerated the reduction of methylene blue (*M*/5000) in the presence of sufficient amounts of the phosphate (*M*/1000). It might be interpreted as suggesting that the redox potential of amine dehydrogenation is higher than that of methylene blue- leucomethylene blue.

The bacterial amine dehydrogenases obtained by us as a cell-free extract, seem to be the first dehydrogenases acting on primary amino group. The bacterial enzymes are very similar to animal and plant amine oxidases, especialy diamine oxidase (*3*) in their prosthetic group (FAD) (*8*), behaviors toward dialysis (*7*) and various inhibitors (*1, 2*) or pH-activity curves (*1, 2*), though animal and plant ones have been assumed to be an oxidase (*4, 5*) and to act on histamine and diamines widely (*3, 4*). The prime difference between two kinds of enzymes, one acting as a dehydrogenase and the other as an oxidase, might be explained by the difference in the biocatalytic systems involved in their over-all reaction. Recently, however, Diazani (*13*) and Francis (*14*) reported that tetrazoliums could be used for demonstrating the activity of amine oxidase in animal liver and kidney. Since the essential reaction here should be dehydrogenation, hydrogen acceptors other than oxygen may be now used in the oxidation of amine by animal oxidase.

In conection with the second difference, the specificity for their substrate, it should be mentioned that the usual animal and plant amine oxidase preparations were so crude that their activity was less than one-fiftith of that of the bacterial one used in the present study. The animal and plant enzyme preparations might be a mixture composed of various amine oxidases and there have been some reports indicating it. Thus Kappler-Adler found that dialysis of aminal histaminase (diamine oxidase) resulted in decreasing the action on histamine while increasing that on putrescine (*8*), and that the ratio of activity on histamine and putrescine of the enzyme preparation from hog kidney (*8*), from the serum of pregnent women (*15*) and from placenta (*16*), differed somewhat. Kenten and Mann (*5*) observed with the enzymes from pea seedlings that the pH-activity curves of histamine- and putrescine-oxidation differed from each other. The fact that bacterial histamine- and putrescine-dehydrogenases were obtained separately might be of significance in connection with the above-mentioned findings.

SUMMARY

1. Two adaptive bacterial enzymes, histamine- and putrescine-dehydrogenases were obtained in a cell-free extract, from the cells of *Achromobacter sp.*, previously adapted to the corresponding amines.

2. The dehydrogenases were found to be flavoproteins, having flavin-adenine-dinucleotide as their prosthetic group, which was rather resistant against dialysis.

3. The inhibition of both enzymes caused by methylene blue, Atabrine, and quinine seemed to be due to their competition with the prosthetic group for the apo-enzymes.

4. The inhibition by these substances was prevented by the previous presence of riboflavin and its 5-phosphate, though their later addition failed to recover the already inactivated enzymes.

5. These results were disccussed in connection with the facts known about animal and plant amine oxidases.

We wish to express our sincere thanks to Prof. K. Miyaki and Prof. T. Yanagita, for their unfailing interest and for many helpful advice and discussion during the course of this work. We also thank Mr. T. Seki for flavin-adenine-dinucleotide offered. A part of the expenditure of this work was defrayed by the Scientific Research Funds from the Ministry of Education, for which grateful acknowledgement is here made.

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THE STRUCTURE OF ADENYLTHIOMETHYLPENTOSE. II*

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(Received for publication, July 23, 1953)

It has been shown in a previous papaer (*1*) that the structure of adenylthiomethylpentose (ATMP) is 9'-(5-thiomethylpentofuranosyl)-adenine, based on periodate titration of ATMP, thiopentitol and thiomethylpentose while the aldoese nature of thiosugar has been evidenced by titration according to the Willstätter-Schudel method (*2*).

The author have now obtained the definite evidence to indicate that the structure of ATMP is 9'-(5-thiomethyl-ribofuranosyl)-adenine (**I**). The experiments which the author have performed are as follows: —In the first place the molecular weight of thiopentitol was determined according to the method of Rast aiming to find out the molecular weight of ATMP, and was proven to have the theoretical value. What, then, is the structure of the thiopentose?

Raymond (*3*) has synthesized a thiomethyl derivative of D-xylose and said that the osazone of this compound (this was said to be 5-thiomethylxylosazone or 5-thiomethyllyxosazone) seemed to differ from that of the natural thiomethylpentose.

In order to confirm this description the author synthesized the thiomethyl derivative of D-xylose according to Raymond (*3*) and established its structure in fact to be 5-thiomethyl-D-xylose by the Willstätter-Schudel titration (*2*) and the periodate oxidation according Malaprade (*4*). As the description of Raymond (*3*), the osazone of this thiosugar differed from that of the natural sugar. This fact excrudes the possibility of the identity of the natural thiosugar with thiomethyl-xylose or -lyxose. By paperchromatography too the synthetic thiomethyl-D-xylose could be difinitely distinguished from the natural sugar. So the two remaining pentoses, namely 5-thiomethylribose and 5-thiomethylarabinose, must be taken into consideration.

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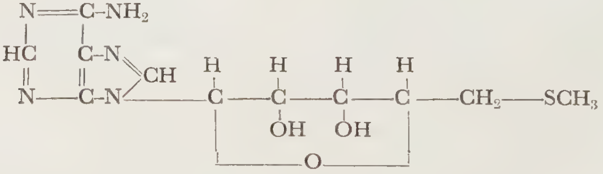
* Preliminarily reported in *Nature*, **165**, 767 (1950)

To settle this question the author applied the Boeseken (*5*) boric acid test to ATMP. The Boeseken test was applied by Makino (*6*) and Lohmann (*7*) to establish the chemical structure of adenosine triphosphoric acid. This structure has been confirmed by Lythoge and Todd (*8*). Adenosine, muscle adenylic acid and adenosine triphosphoric acid give a positive reaction, while for nucleotides from yeast nucleic acid the reaction is negative. Glucose, which gives a positive reaction, becomes negative when combined into saccharose, indicating that the *cis*-hydroxyls of glucose are lost by the formation of a δ-oxide ring. In the case of ATMP, the Boeseken test was as strongly positive as that of adenosine. The two hydroxyls at positions 2 and 3 of the thiosugar are not only uncombined but also arranged to the *cis*-position, indicating that the thiosugar is a derivative of ribose. As to the combining position of the thiomethylpentose in adenine, there is a study of Falconer and Gulland (*9*) who stated by spectrophotometric researches that the thiosugar is attached in position 9 of the base as in case of adenosine. In order to confirm their description the author made also comparison of its absorption spectra with that of adenosine and came to the same conclusion as that and Falconer and Gulland (*9*). Comparing these results with those obtained from periodate oxidation of ATMP, the author assigns to the latter substance, the accompanying structure 9'-(5-thiomethylribofuranosyl)-adenine (**I**).



(**I**)

EXPERIMENTAL

Determination of Molecular Weight of Thiopentitol—

Weight of thiopentitol 1.130 mg., m.p. 117°
Weight of camphor 12.980 mg., m.p. 178° K=40
Melting point depression Δt:178.0°–159.0°=19.0°
Found M=183.2 ($M=K\cdot 1000\cdot S/L\cdot\Delta t$)
Calcd. for $C_6H_{14}SO_4$:182.17

*5-Thiomethyl-*D*-xylose*—The monoacetone-D-xylose was prepared from D-xylose according to Svanberg and Sjöberg (*10*). The monoacetone tosyl-D-xylose was obtained by heating it with *p*-tosylchloride, and converted to 5-thiomethyl monoacetone D-xylose by treating with sodium methylmercaptan.

After hydrolysis the resultant thiomethyl-D-xylose was isolated according to Raymond (*3*).

Periodate Oxidation of 5-*Thiomethyl-*D*-xylose*—4.85 mg. of 5-thiomethyl-D-xylose were treated with 3 ml. of 0.05 *M* potassium periodate solution and then titrated as usual: 9.90 ml. of 0.1 *N* sodium thiosulfate were used which correspond to the consumption of 3.9 moles of oxydant.

*Willstätter-Schudel's Titration of 5-Thiomethyl-*D*-xylose*—4.85 mg. of 5-thiomethyl-D-xylose were mixed with 2.00 ml. of 0.1 *N* iodine and 1 ml. of *N* sodium hydroxide solution and, after allowed to stand at room temperature for 10 hours, the mixture was acidified with sulfuric acid and then the liberated iodine was titrated with 0.1 *N* sodium thiosulfate solution: 0.86 ml. of 0.1 *N* sodium thiosulfate were used, which correspond to the consumption of 1.96 moles of iodine, indicating that both an aldehyde and thiomethyl group are simultaneously oxidized.

Determination of Formic Acid after Periodate Oxidation—4.9 mg. of 5-thiomethyl-D-xylose dissolved in 2 ml. of water were oxidized with 35.0 mg. potassium periodate and after 5 hours the formic acid produced was titrated with 0.04 *N* sodium hydroxide solution using methylred as an indicator: 2.00 ml. of 0.04 *N* sodium hydroxide were used, corresponding to 2.9 moles of formic acid.

Phenyl Osazone of Thiosugar from ATMP—50 mg. of ATMP were hydrolysed by heating with 1 ml. of *N* sulfuric acid solution in a sealed tube in a boiling water bath for 1 hour. After cooling the separated adenine was filtered off, and to the filtrate was added 0.05 ml. of phenylhydrazine dissolved in 0.05 ml. of glcial acetic acid. The osazone which separated out was filtered and recrystallized from methanol. It was a felt of coarse melon-yellow needles melting at 158–159°.

*5-Thiomethyl-*D*-xylosazone*—20 mg. of 5-thiomethyl xylose were dissolved in a small amount of water, and 50 mg. of phenyl hydrazine hydrochloride and 5 mg. of sodium acetate, dissolved in a small amount of water, were added to it. The subsequent treatment was the same as described for the thiomethylpentosazone from the natural product. 5-Thiomethyl-D-xylosazone which separated was a felt of coarse lemon-yellow needles and on heating it softened slightly at 150°, and melted at 162° and showed a marked depression (15°) when admixed with the phenyl osazone of thiosugar from ATMP.

Paperchromatography of Thiosugars—The thiosugar from the natural product and the synthetic 5-thiomethyl-D-xylose were chromatographed on filter paper No. 50 of Tōyō-Roshi using the ascending method with water-saturated normal butanol as the developer, and detected with ammoniacal silver nitrate solution or platinum reagent (*11*). The Rf value of 5-thiomethyl-D-xylose was 0.67 and could be separated from that of the natural thiomethylpentose indicating 0.62. Thus there can be no doubt as to the non-identity of the two sugars.

Boeseken's Boric Acid Reaction of ATMP (*5*)—10 ml. of 0.5 *M* boric aic solution were mixed with 7.5 ml. of 0.1 *N* sodium hydroxide solution and 50 drops of alcoholic solution of 1% phenolphthalein and brought into a volumetric flask of 100 ml. and the whole was filled up to 100 ml. with water. To the solution of test substance (0.2 ml.) containing a drop of alcoholic solution of phenolphthalein was added 0.01 *N* sodium hydroxide until the solution became slightly pink similar to the color of the boric acid solution prepared as described above, and to this solution of the test substance 0.2 ml. of the boric acid solution was added. If the color of the mixture disappeared 0.01 *N* sodium hydroxide solution was again added until it regained the original pink color. Among the test substances, besides ATMP, adenosine and thiopentitol, were chosen polyalcohols, 3- and 5-nucleotides as control. In the case of ATMP the Boeseken test was as strongly positive as that of adenosine, while for 3-nucleotides the reaction was negative.

TABLE I

Boeseken Boric Acid Test

Substances	H_3BO_3 : Substance =1 mole : 1 mole		H_3BO_3 : Substance =1 mole : 2 moles		Results
		0.01 *N* NaOH used		0.01 *N* NaOH used	
	mg.	*ml.*	*mg.*	*ml.*	
Mannitol	1.82	0.72	0.91	0.40	(+)
Adonitol	1.52	0.39	0.76	0.24	(+)
Adenosine	2.76	0.54	1.38	0.35	(+)
ATMP	2.97	0.54	1.48	0.35	(+)
Thiopentitol	1.82	0.36	0.91	0.22	(+)
Adenylic acid-3	3.65	—	1.82	—	(−)
Adenylic acid-5	3.47	0.52	1.74	0.34	(+)
Cytidylic acid-3	3.23	—	1.62	—	(−)
Glucose	1.80	0.65	0.90	0.40	(+)
Saccharose	3.42	—	1.71	—	(−)

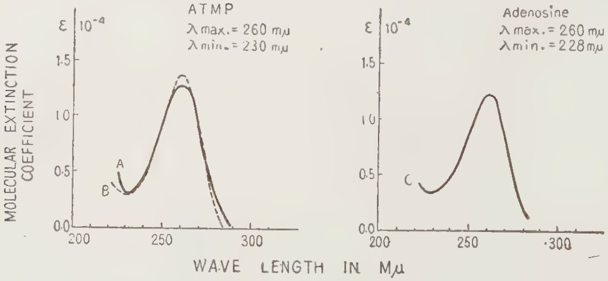


FIG. 1. Ultra violet absorption of ATMP and adenosine

A. ATMP in water and 0.05 *N* HCl.
B. ATMP in 0.05 *N* NaOH at 2 hrs. after preparation of the solution.
C. Adenosine in water, 0.05 *N* HCl and 0.05 *N* NaOH.

Ultra-violet Absorption Spectra—The instrument used was the Shimazu quartz spectrophotometer model QS-20, photograph being taken on the Fuji process film using double sector method (number of rotations beyond 10/sec.) with condensed spark from iron poles as the light source through a slit of 3/100 mm.

The results were illustrated in Fig. 1: as can be seen from it the ultra-violet absorption curve of ATMP was quite similar to that of adenosine.

We are indebted to Dr. S. Ohdake for a gift of adenylthiomethylpentose and to Prof. T. Mori for a gift of thiopentitol.

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THE STRUCTURE OF ADENYLTHIOMETHYLPENTOSE. III*

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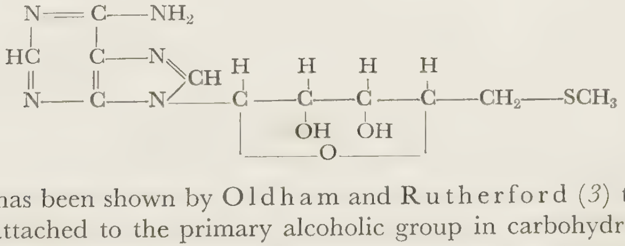
Adenylthiomethylpentose (ATMP) is a sulfur containing, characteristic nucleoside first isolated from yeast and crude oryzanine. On hydrolysis it yields adenine and a thiomethylpentose. In the previous paper (*1*), the thiosugar was recongnized as an aldose on the basis of titration data obtained by the Willstätter-Schudel method. Studies on periodate oxidation of the ATMP, the thiosugar and the thiopentitol obtained from it by reduction with sodium amalgam, indicated that ATMP is 5-thiomethylfuranopentoside (*1*). The thiosugar and its osazone were shown not to be identical with 5-thiomethyl-D-xylose and its osazone which were synthesized according to Raymond, and the structure of which were determined by the present author (*2*). The positive Böseken test of ATMP favoured the ribose configuration similar to that of adenosine. The nucleoside must then be a derivative of 5-thiomethylribose. Spectroscopic evidence favoured N_9 for the connecting position of sugar to adenine. On the basis of these evidences, the structure of ATMP is considered to be 9'-(5-thiomethyl-D-ribofuranosyl) adenine (**I**).


(**I**)

It has been shown by Oldham and Rutherford (*3*) that a tosyl group attached to the primary alcoholic group in carbohydrate derivative is readily substituted with iodine on treatment with sodium iodide in acetone. The applicability of this reaction has since been extended to the study of the pentofuranoside by Levene *et al.* (*4*).

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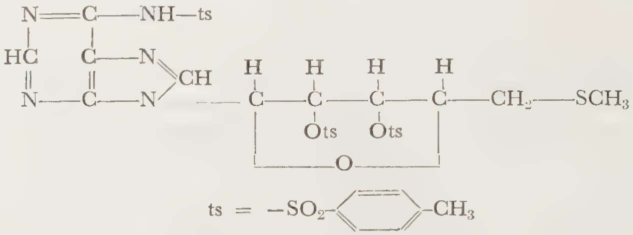
* Preliminarily reported in Nature, **167**, 238 (1951)

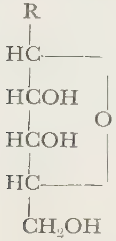
Now ATMP gave a tritosyl derivative on treatment with 3.3 moles of *p*-tosyl chloride in dry pyridine. As none of its tosyl residues of this compound was replaced by iodine on treatment with sodium iodide in acetone during 2 hours at 100°, it follows that there is no tosyl group at position 5 of the sugar portion, and so one of these must be attached to the amino group of the purine base, and two attached to the secondary alcoholic groups of the sugar.

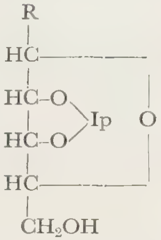


(II)

Monoacetone adenosine, ((**IV**)R'=NH_2) obtained by condensing adenosine with acetone in the presence of zinc chloride by the method of Levene and Tipson (*5*), was converted to 5-tosyl-monoacetone adenosine ((**V**)R'=NH_2) by treating with *p*-tosyl chloride in dry pyridine. Dry tosyl monoacetone adenosine ((**V**)R'=NH_2) and dry sodium methyl mercaptan were dissolved in acetone and the solution heated in a sealed tube at 100°. The reaction product (5-thiomethylmonoacetone adenosine) after conversion into the picrate, which had a melting point of 172–174° (decompn.), was compared with the monoacetone derivative of the natural compound and showed no melting point depression when admixed with the picrate of monoacetone ATMP, and, on the paper chromatogram, gave the same Rf value (0.52 in butanol-water) as the latter. The isopropylidene group of 5-thiomethylmonoacetone adenosine was hydrolysed by normal sulfuric acid to give 5-thiomethyladenosine again, which showed the same Rf value (0.59 in butanol-water) as the natural ATMP on the paper chromatogram. Since the structure of adenosine has been established by synthesis and the configuration of it glycosidic centre has been confirmed to be β-D-type (*6*) it follows that the structure of ATMP is 9'-(5-thiomethyl-β-D-ribofuranosyl) adenine (**I**).

Also, from 5-tosyl-2,3-isopropylidene inosine ((**V**)R'=OH), 5-thiomethyl-2,3-isopropylidene inosine ((**VI**)R'=OH) was obtained by heating with sodium methyl mercaptan in acetone. The reaction product was converted into a picrate. When treated with nitrous acid ATMP gave hypoxanthinyl thiomethylpentose (*7*), which yielded a monoacetone derivative on condensing with acetone. The picrate of the latter had a melting point 105°, and showed no melting point depression when admixed with the picrate of 5-thiomethyl monoacetone inosine.


(III)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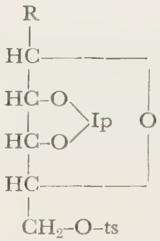
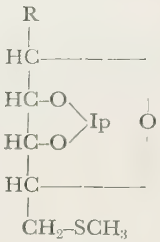
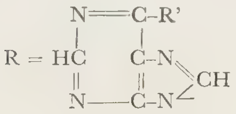
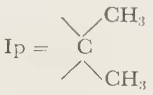

(V)


(VI)



R'{ $-NH_2$ (Adenine)
  $-OH$ (Hypoxanthine)



EXPERIMENTAL

Tritosylation of ATMP—297mg. of dry ATMP were dissolved in 3cc of dry pyridine, and 628 mg. of dry, finely powdered tosyl chloride were added to the solution, and the mixture was shaken until the chloride dissolved. After standing overnight at room temperature with the exclusion of atmospheric moisture, 0.3 ml. of water was added to the brown solution, and the reaction mixture was kept at room temperature during 30 minutes, and then ice-cold saturated sodium bicarbonate solution was added until no more effervescence occured and then extracted three times with chloroform (20 ml., 20 ml. and 10 ml., respectively). The chloroform extracts were united, dried over anhydrous sodium sulfate, filtered, and the filtrate evaporated to a syrup. The product dried in the vacuum desiccator gave after several days about 0.5 g. of a pale brown, crystalline mass, which was dissolved in 3 ml. of acetone and precipitated with ether. After filtration of brown flocculent precipitate, the filtrate was evaporated to dryness *in vacuo* giving a pale yellow brown crystalline mass, which melted with decomposition at 158–160°. The yield was 425 mg.

Calcd. for $C_{32}H_{33}O_9N_5S_4$	(759.18)	N 9.21
Found		N 9.27

Action of Sodium Iodide on Tritosyl ATMP—A mixture of 100 mg. of dry tritosyl ATMP and 200 mg. of dry sodium iodide was dissolved in 20 ml. acetone and the solution was heated in a sealed tube at 100° for 2 hours. No trace of crystalline substance separated out during this treatment.

Preparation of Crystalline Monoacetone Adenosine—100 mg. of finely powdered adenosine (dried at 120° under diminished pressure for 48 hours) were dissolved in filtered solution of 300 mg. of anhydrous zinc chloride in 3 ml. of dry acetone. The solution was boiled under a reflux condenser with the exclusion of atmospheric moisture. The clear pale yellow solution was allowed to stand overnight at room temperature with the exclusion of atmospheric moisture and was then evaporated under diminished pressure to a small volume. Dry ether (3 ml.) was added to it with shaking and the white flocculent precipitate was filtered off, and dried in the vacuum desiccator. The acetone-ether mother liquor was evaporated to dryness and dried in a high vacuum giving a thick syrup. This was again treated with 3 ml. of dry ether and the white flocculent precipitate filtered off and dried in the vacuum desiccator.

The two portions of ether-insoluble material were dissolved in 8 ml. of distilled water. A boiling saturated solution of barium hydroxide was now rapidly added, until the precipitate which first formed just redissolved. A few drops of octyl alcohol were added and then carbon dioxide was passed into the solution until it became neutral (pH 7.0 to B.T.B., Tōyō Roshi). The precipitate, which was readily filtered off, was air-dried and extracted with boiling absolute methanol and then with boiling water. The filtrate, methanol extracts and aqueous extracts were evaporated to dryness with a fan. The three portions of white powdery product were combined and extracted with boiling acetone in a small Soxhlet apparatus for 7 hours. The acetone extracts were evaporated to dryness *in vacuo.*, giving a colorless crystalline mass of mono acetone adenosine. Monoacetone adenosine was recrystallized from absolute methyl alcohol, melting with decomposition at 200° (with slight softening at 190°), Yield 60 mg.

Tosylation of Monoacetone Adenosine—60 mg. of dry monoacetone adenosine were dissolved in 1.0 ml. of dry pyridine and, 42 mg. of dry, finely powdered tosyl chloride were added, and the mixture was heated to about 60° under a reflux condenser closed by a calcium chloride tube, until all the chloride had dissolved. The solution was then cooled to room temperature, allowed to stand, with the exclusion of atmospheric moisture, for 4 days at room temperature. The brown solution was heated to about 60° during 10 minutes and then cooled to room temperature. 0.1 ml. of water was added to it and the mixture was kept at room temperature for 30 minutes and was cooled to 0° in ice-salt mixture, and then a cold, saturated aqueous solution of sodium bicarbonate was added until there was seen no more effervescence and the solution became slightly alkaline (pH 7.6 proved by B.T.B., Tōyō Roshi), and then diluted with 2 ml. of water and extracted three times with chloroform (10 ml., 10 ml. and 10 ml. respectively); the chloroform extracts were united, dried with anhydrous sodium sulfate, filtered, and the filtrate was evaporated to dryness under diminished pressure. The resultant product was dried in a vacuum desiccator over sulfuric acid until free from pyridine odour, giving a slightly brown mass. Weight 50 mg., m.p. 199–200°.

Calcd. for $C_{20}H_{23}O_6N_5S$ (461.29) N 15.18

Found N 15.52

5-Thiomethyl Monoacetone Adenosine—

a) 44.7 mg. of dry monoacetone monotosyl adenosine, 13 mg. of dry sodium methyl mercaptan (*8*), and 0.5 ml. of dry acetone were heated in a sealed tube in the boiling water bath for 2 hours. After cooling to room temperature, the tube was opened and washed with a small amount of acetone. A trace of acetone-insoluble substance was filtered off and the filtrate concentrated *in vacuo* to a brown syrup.

b) The brown, syrupy reaction product (5-thiomethyl monoacetone adenosine) was treated with an etheral solution of picric acid. The picrate which settled out was then filtered, washed with a little of ether, dried on a pottery plate. It melted with decomposition at 174°.

Calcd. for $C_{14}H_{19}O_3N_5S{\cdot}C_6H_3N_3O_7$ (566.31) N 19.7 S 5.6

Found N 19.3 S 5.3

Preparation of Monoacetone ATMP—

a) 400 mg. of dry finely powdered ATMP were dissolved in a filtered solution of 1.6 g. of anhydrous zinc chloride in 15 ml. of dry acetone and the reaction product isolated in the same manner as described for monoacetone adenosine.

m.p. 143° (decomp. 190–192°)

Calcd. for $C_{14}H_{19}O_3N_5S$ (337.26) N 20.7

Found N 19.9

b) Picrate: The procedure to obtain it was the same as for the preparation of the picrate of 5-thiomethyl acetone adenosine, m.p. 174–175°.

Calcd. for $C_{14}H_{19}O_3N_5S{\cdot}C_6H_3N_3O_7$ (566.31) N 19.78 S 5.66

Found N 19.76 S 5.8

It showed no melting point depression when admixed with the picrate of monoacetone thiomethyl adenosine.

Paper Chromatography of the Picrate of 5-Thiomethyl Monoacetone Adenosine and the Picrate of Monoacetone ATMP—The synthetic picrate of 5-thiomethyl monoacetone adenosine and the picrate from natural material were chromatographed on filter paper, No. 50 of Tōyō Roshi, with water-saturated *n*-butanol as the developer, using ascending methode, and the spots on the paper strip were observed under the ultraviolet lamp. The spots of these two substances showed the same Rf value (0.52).

Hydrolysis of Monoacetone ATMP—Monoacetone ATMP was added to *N* sulfuric acid and slightly warmed at 60°. The solution was cooled at room temperature and, after one hour, barium carbonate was added to remove sulfuric acid and filtered. The filtrate was allowed to stand in the refrigerator overnight. The colorless crystalline needles which separated were filtered and recrystallized from water. It melted at 210° and showed no melting-point depression when admixed with the natural ATMP and gave the same Rf value (0.59) as that of the latter compound on paperchromatogram when observed under ultraviolet lamp.

The source of ultra-violet light used for the detection of the purine on filter paper was a germicidal lamp (Matsuda GL-15, 100 v. A. C., 15 watt), the light from which was filtered through two quartz cells, both having 30 mm. thickness, one of which contained dry chlorine and the other a nickel and cobalt salt solution (350 g. of $NiSO_4 \cdot 7H_2O$ and 100 g. of $CoSO_4 \cdot 7H_2O$ were dissolved in 1 l.) (*9*).

Preparation of Monoacetone Inosine—800 mg. of granulated anhydrous zinc chloride were quickly transfered to a flask and treated with 20 ml. of dry acetone. The small amount of white insoluble material was filtered off, and to the clear, colorless filtrate were added 200 mg. of dry finely powdered inosine and the reaction product was isolated according to Levene and Tipson (*10*). m.p. 240–245°, yield 70 mg.

5-*Tosyl-monoacetone Inosine*—To 70 mg. of dry monoacetone inosine, dissolved in 2 ml. of dry pyridine, were added 50 mg. of tosyl chloride, and the reaction product was isolated as described by Levene and Tipson (*10*). m.p. 195°

Calcd. for	$C_{20}H_{22}O_7N_4S$ (462.28)	N 12.13
Found		N 12.29

5-*Thiomethyl Monoacetone Inosine*—

a) 23 mg. of 5-tosyl monoacetone inosine, 7 mg. of dry sodium methyl mercaptane, and 0.4 ml. of dry acetone were heated in a sealed tube in a boiling water bath for 2 hours. After cooling to room temperature, the tube was opened and washed with a small amount of acetone. A small amount of acetone-insoluble matter was filtered off, and the filtrate was concentrated *in vacuo* to a brown grassy mass.

b) This crude reaction product (5-thiomethyl monoacetone inosine) was treated with an ether solution of picric acid. The picrate which settled out was then filtered, washed with a small amount ether, dried on a pottery plate. It melted with decomposition at 102° (rapidly heated).

Calcd. for	$C_{14}H_{18}O_4N_4S \cdot C_6H_3N_3O_7$ (567.29)	N 17.28
Found		N 16.4

Hypoxanthinyl Thiomethylpentose—0.2 g. of dry ATMP were suspended in 7 ml. of distilled water and 0.4 g. of barium nitrite monohydrate were added. The mixture was cooled to 0° and 0.3 ml. of glacial acetic acid were added to the solution. After allowed to stand at room temperature for one week in the dark, with the exclusion of dust, the solution was now evaporated *in vacuo* at 35° to 2 ml. and was allowed to stand in the refrigerator overnight. The crystalline mass which separated was filtered off. The dilute sulfric acid was added to the filtrate until the solution was slightly acid to congo red, and then barium hydroxide solution was immediately added until this mineral acidity was exactly neutralized. The barium sulfate was removed by centrifugation, and the supernatant evaporated to dryness under diminished pressure, giving a white crystalline mass, which melted with decomposition at 235° (with slight softening at 220°).

Picrate, m.p. 134°

Calcd. for	$C_{11}H_{14}O_4N_4S \cdot C_6H_3N_3O_7$ (527.26)	N 18.6
Found		N 16.9

Monoacetone Hypoxanthinyl Thiomethylpentose—200 mg. of dry, crude hypoxanthinyl thiomethylpentose were dissolved in a filtered solution of 0.8 g. of anhydrous zinc chloride in 20 ml. of dry acetone, and the reaction product was isolated as described for the preparation of monoacetone adenosine.

Picrate, m.p. 102–105°
Calcd. for $C_{14}H_{18}O_4N_4S \cdot C_6H_3N_3O_7$ (567.29) N 17.28
Found N 16.75

The picrate of synthetic 5-thiomethyl monoacetone inosine showed no melting point depression when admixed with the picrate of monoacetone hypoxanthinyl thiomethylpentose from the natural source and gave the same Rf (0.48) on paper chromatogram (butanol-water) as that of monoacetone hypoxanthinyl thiomethylpentose.

The author wishes to thank Prof. Dr. Katashi Makino for his kind guidance.

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TABLE I

Effect of Acetone Treatment upon Activity of Glucose Dehydrogenase

	Enzyme 1*	Enzyme 2**
Optical density	0.06	1.50
Decolorization time of methyleneblue (1 : 500)† *minutes*	>180	9∼10

* Enzyme 1. Equal volume of acetone was added to glucose dehydrogenase solution prepared as described in text: the mixtures left standing for 2 hours at 0°, and then centrifuged; the precipitates dissolved in 0.1 *M* phosphate buffer, the total volume was equal to original volume.

** Enzyme 2. Enzyme solution regulary prepared.

† Methylene blue was used in place of TTB, other conditions were similar to conditions shown in text.

Each value above described is the mean value of five experiments.

between 33 and 50 per cent saturation. The precipitate was dissolved in a small amount of water, and dialyzed first against running tap water overnight and then against a 100-folds volume of distilled water in an ice-chest for one day.

Precipitate formed during dialysis was discarded, and a dark reddish brown solution obtained was used in the following experiments. It was storable in a frozen state without much loss of activity for about 3 months.

Preparation of DPN (Coensyme I)—DPN was prepared from baker's yeast by the method of Williamson and Green (*6*). The purity of the preparation with respect to DPN amounts to 40∼50 per cent according to the estimated values of total organic phosphorous.

Preparation of Straub's Flavoprotein—Flavoprotein was purified from pig heart muscle chiefly by Straub's method (*7*), though the aluminium hydroxide treatment and the second ammonium sulfate fractionation were ommitted.

Methods

Glucose dehydrogenase activity was measured as follows, except otherwise stated: Place in the side arm cap of a Thunberg tube 0.5 ml. of freshly prepared 0.5 per cent TTB solution and either 0.5 ml. of 2 *M* D-glucose solution or distilled water in the case of a blind test; place in the tube 1 ml. of glucose dehydrogenase solution, 0.5 ml. of 1 per cent DPN solution, 0.5 ml. of flavoprotein solution, 1 ml. of 0.2 *M* phosphate buffer (pH 7.4) and, if necessary, other chemicals. The total volume of reaction mixtures was always made to be 5 ml.

All the Thunberg tubes containing the reaction mixtures were simultaneously evacuated to 10 mm.-Hg. with an oil pump and left then for 3 minutes, then flushed with pure nitrogen gas and further evacuated again to 10 mm.-Hg. The same treatment was repeated thrice. The Thunberg tubes were set in the water bath (37°), the substrate and TTB were tipped into the tubes and the reaction was allowed to proceed usually for 15 or 30 minutes. The side arm caps were then opened and the reaction was stopped by a rapid addition of 5 ml. of glacial acetic acid into each tube. After adding 5 ml. of toluene and shaking vigorously, the mixtures were centrifuged (4000 r.p.m., for 5 minutes). The color densities of supernatant clear deep-red formazan solutions separated on the upper surface of coagulated protein were measured photometrically by Purflich's photometer or Beckman's spectrophotometer -DU. A control measurement was made using water instead of the glucose solution. By the enzymic reduction of TTB, only one kind of dye was produced.

RESULTS

Relationship between Formazan Concentration and Optical Density—The formazan solution in toluene showed its maximal light absorption at a wave length of 495 mμ and this wave length was therefore employed in all of the following colorimetric measurements. A linear relationship was recognized between the optical density and the concentration of formazan in toluene (Fig. 1). The formazan produced enzymatically from TTB was fairly stable in toluene and thus, when allowed to stand aerobically at room temperature, no measureable changes in optical density were observed in about 2 hours.

Relationship between Reaction Time and Optical Density—The rate of enzymatic reduction of TTB under the above conditions was investigated. In the middle course of the reaction, a pretty linear relationship was observed between reaction time and optical density as illustrated in Fig. 2. It was not the case when the reaction was sought for under aerobic conditions or mere evacuated conditions.

Effect of Enzyme Concentration—The reaction velocity was proportional to the enzyme concentration as illustrated in Fig. 3. TTB was not reduced by heat-inactivated enzyme under the same condition as above described.

Effect of Substrate Concentration—The substrate concentration was varied from 0 to 0.4 moles. The results were shown in Figs. 4 and 5. Michaelis constant (K_m) derived from the results by Michaelis-Menten's method (*8*) and Lineweaver-Burk's (*9*) was 0.075 and 0.085 *M* respectively. A maximal reaction rate was obtained at the substrate concentration of about 0.28 *M*, which corresponds to the values reported by Strecker and Korkes (*10*).

Effect of DPN Concentration—It was ascertained that DPN was necessary to link glucose dehydrogenase to other terminal hydrogen

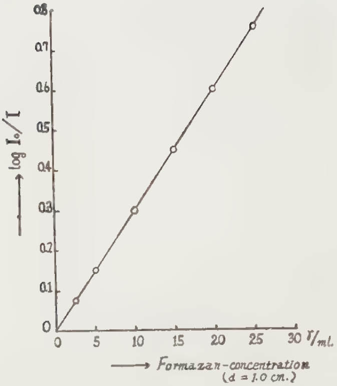

Fig. 1. Relationship between formazan concentration and optical density. For details see text.

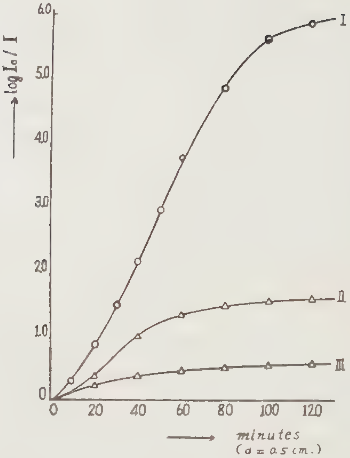

Fig. 2. Effect of oxygen upon the velocity of the glucose dehydrogenase reaction.

I. Evacuated and substituted with nitrogen gas,
II. Only evacuated with water pump,
III. Aerobic condition.

acceptors, such as flavoprotein and TTB (Table II). The optimal concentration of DPN in the determination system was found to be 0.1 per cent under the above conditions.

Effect of Straub's Flavoprotein Concentration—In 1952 Brodie and Gots (*11*) claimed that the reduction of tetrazolium salts in vitro by isolated bacterial enzymes could be accomplished by the use of flavin enzyme, and neither DPN-linked dehydrogenase nor reduced DPN could reduce the tetrazolium salt to formazan. Adler and Euler (*12*), and Ogston and Green (*13*) showed that yeast flavoprotein could transfer hydrogen from glucose dehydrogenase to molecular oxygen. Wainio and Eichel (*14*) also obtained the same results by the use of heart flavoprotein as hydrogen carrier. It has been investigated now whether flavoprotein should be necessary to link DPN to TTB or not.

From the results given in Table III, it appears that although the addition of flavoprotein caused an increase in the activity of glucose dehydrogenase, flavoprotein might not be absolutely indispensable as a hydrogen carrier, but it is also probable that the enzyme preparation might contain a small amount of flavoprotein as impurity. The effect

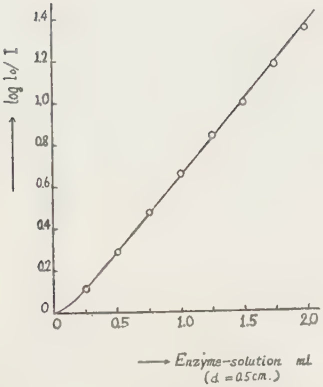

FIG. 3. Relationship between color developed and quantity of enzyme solution. (For details, see text).

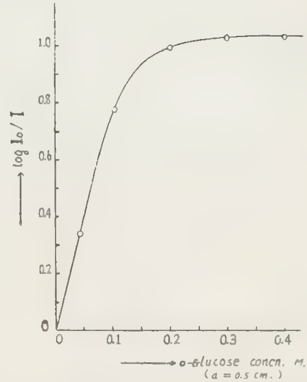

FIG. 4. Effect of substrate concentration upon the activity of glucose dehydrogenase.

The Michaelis constant (K_m) of glucose dehydrogenase-glucose system was determined by the method of Michaelis-Menten, as 0.075 *M* at 37°. (For details, see text).

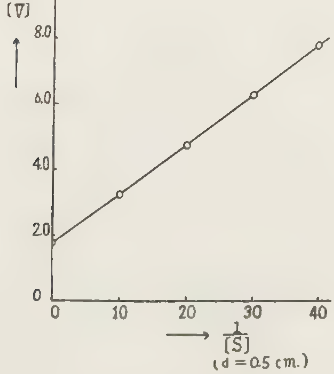

FIG. 5. The reciprocal of the initial velocity of the glucose dehydrogenase reaction plotted against 1/(S).

The Michaelis constant (K_m) of glucose dehydrogenase-glucose system was determined by the method of Lineweaver-Burk, as 0.085 *M* at 37°.

TABLE II

Effect of DPN Concentration upon the Activity of Glucose Dehydrogenase

DPN concentration*	Optical density
mg.	
0.0	0.0
1.0	0.28
2.5	0.35
5.0	0.41
7.5	0.51
10.0	0.55

(d=0.5 cm.)

* The value of DPN-concentration was demonstrated in terms of total weight used in each tube.
(For details see text).

TABLE III

Effect of Flavoprotein upon the Activity of Glucose Dehydrogenase

Flavoprotein soln. added	Optical density
ml.	
0.0	0.30
0.25	0.42
0.50	0.52
0.75	0.62
1.00	0.68
1.50	0.74
2.00*	0.79

(d=0.5 cm.)

* In this case, total volume of reaction mixtures was made to 5.5 ml. The reaction mixtures contain, 1.0 ml. of 0.2 *M* phosphate buffer, 1.0 ml. of enzyme solution, 0.5 ml. of 1 per cent DPN solution, 0.5 ml. of 2 *M* D-glucose, 0.5 ml. of 0.5 per cent TTB solution and 2 ml. of flavoprotein.

of flavoprotein in case of a purified enzyme preparation will be reported later.

DISCUSSION

Recently, colorimetric techniques using tetrazolium salts as a hydrogen acceptor have been widely employed in the study of many enzyme systems, since they can be performed under aerobic conditions without the use of Thunberg tubes. But their quantitative applications to the single definite dehydrogenase system have been seldom attempted (*11*, *15*, *16*). From the results of the study on glucose dehydrogenase given above, it is clear that oxygen hinders the reduction of TTB by the enzyme. The effect of oxygen may be due to the competitive action of the oxidase system for electrons. There is, however, another possibility, as reported by Wainio and Eichel (*14*), that flavoprotein, when oxidized, may produce an amount of hydrogen peroxide, which is sufficient to decrease the rate of the reaction. It is difficult to decide which is the actual figure in the present experiments, because the enzyme solution used was found to contain some catalase activity (Table IV) and may probably have contained oxidases as impurity.

TABLE IV

The Determination of Catalase Activity of Glucose Dehydrogenase Preparation

t	0.04 M $KMnO_4$ (a—x)	k
seconds	*ml.*	
0	5.50	
5	4.84	0.0111
10	4.22	0.0115
15	3.66	0.0117
20	3.20	0.0117
30	2.50	0.0114

Catalase activity of the glucose dehydrogenase preparation was determined by the drop method contributed by Shirakawa (*17*).

SUMMARY

1. A colorimetric quantitative determination of liver glucose dehydrogenase activity was attempted by use of 2,3,5-triphenyltetrazolium bromide as hydrogen acceptor.

2. Reduction of TTB was carried out anaerobically in Thunberg tubes.

3. At pH 7.4, the Michaelis constant (K_m) of the enzyme was 0.075 or 0.085 M according as Michaelis-Menten's or Lineweaver-Burk's method was employed.

4. DPN was necessary as hydrogen carrier between glucose dehydrogenase and terminal hydrogen acceptors.

5. It may appear that flavoprotein (Straub's) may act as hydrogen carrier between reduced DPN and TTB which functions as a hydrogen acceptor.

The author is indebted to Dr. Y. Oshima and Dr. M. Shirakawa, of the Laboratory of Biochemistry, Faculty of Agriculture, and Dr. T. Misao, of the First internal Clinic, Faculty of Medicine, Kyushu University, who suggested the problem and gave many valuable advices.

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BACTERIOSTATIC ACTIVITY OF SOME PYRIDINIUM, NICOTINIUM, QUINOLINIUM, AND ISOQUINOLINIUM QUATERNARY SALTS ON MYCOBACTERIUM TUBERCULOSIS H37Rv[1]

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The effect of surface active agents on microorganisms has been widely studied in recent years. The literature generally indicates that the quaternary ammonium compounds are effective against tubercle bacilli as bacteriostatic agents, but are poor bactericidal agents (*1*, *2*, *3*, *4*, *5*, *6*). Bacteriostatically the quaternary ammonium compounds are generally effective at a concentration of $1:10^4$ to $1:10^5$, and a few are found to be effective at $1:10^6$ (*7*). A pyridinium salt, 3-hydroxy-4-methyl-1-(2,3-dihydroxypropyl)-pyridinium chloride was reported to require a concentration of greater than 64 mg. per cent (saturated solution) for bacteriostatic effect against *M. tuberculosis* H37Rv (*8*). The present paper is the result of a study on a series of pyridinium, nicotinium, and quinolinium quaternary salts.

EXPERIMENTAL

A weighed portion of a 3 to 4 week culture of *Mycobacterium tuberculosis* H37Rv[2] grown on Lowenstein-Jensen's solid medium was ground in a few drops (1/20 of final volume) of 1/4 per cent NaOH, and diluted to appropriate volume with sterile distilled water. The solutions of the compounds tested were made up in sterile distilled water in ten times the test concentrations ($1:10^4$, $1:10^5$, $1:10^6$). Half ml. portions of these solutions were pipetted into 20 ml. screw cap vials containing 4.5 ml. of sterile Proskauer-Beck liquid synthetic medium (*9*). The vials were inoculated with 0.1 ml. of a suspension containing 0.1 mg. wet weight of bacilli, and incubated at 37° for 6 weeks. They were checked for growth at weekly intervals.

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[2] Kindly supplied by William Steenken, Jr. of Trudeau Foundation, Trudeau, New York.

Six vials per dilution of each compound were inoculated. Five of the vials were incubated, while the sixth was refrigerated as a blank for comparison in judging growth. The same number of control vials containing 0.5 ml. of sterile distilled water in place of the chemical solution was included with each test group. The amount of growth was judged visually by comparison with the blanks and the controls.

RESULTS

The compounds tested and the results obtained are presented in the accompanying tables. For the calculation of the inhibition index each vial showing complete inhibition was assigned a value of 2, and each showing partial inhibition, a value of 1. The index as recorded in the tables is the sum of these values for the five vials run for each dilution. Thus, an index of 10 indicates that complete inhibition was obtained in all five vials in the group, while 0 indicates that no inhibition was obtained in any of the vials, and intermediate values indicate that complete or partical inhibition was obtained in varying number of the five vials.

Table I

Bacteriostatic Action of Quaternary Nicotinium Salts on Mycobacterium Tuberculosis H37Rv

Quaternary salt with description of compound[3]	Inhibition index Dilution $1:10^4$	$1:10^5$	$1:10^6$
1. Methylnicotinium bromide Dark brown syrup	0	1	0
2. Ethylnicotinium bromide Dark brown syrup	0	0	0
3. Butylnicotinium bromide Dark brown syrup	0	1	0
4. Octylnicotinium iodide Dark brown syrup Insol. residue—14.4 mg./g.	8	0	0
5. Dodecylnicotinium bromide Dark brown syrup	10	8	0
6. Hexadecylnicotinium bromide Dark brown syrup	10	9	3
7. Octadecylnicotinium bromide Dark brown syrup	10	8	0
8. Octadecylnicotinium laurate Dark brown syrup Insol. residue—24.0 mg./g.	10	10	1

TABLE I (concluded)

Quaternary salt with description of compound[3]	Inhibition index Dilution		
	1:10^4	1:10^5	1:10^6
9. Octadecylnicotinium laurate Dark brown syrup	10	6	0
10. Benzylnicotinium bromide Dark brown syrup Insol. residue—28.8 mg./g.	0	0	0
11. *o*-Chlorobenzylnicotinium bromide Red-brown paste Insol. residue—24.8 mg./g.	0	0	0
12. *p*-Chlorobenzylnicotinium chloride Dark brown syrup Insol. residue—25.2 mg./g.	9	1	0
13. 2,4-Dichlorobenzylnicotinium chloride Brown lumpy solid Insol. residue—25.2 mg./g.	10	0	0
14. 3,4-Dichlorobenzylnicotinium chloride Dark brown syrup Insol. residue—57.6 mg./g.	10	0	0
15. *p*-Nitrobenzylnicotinium bromide Tan colored crystals	1	1	0
16. Dimethylnicotinium dibromide White crystals	0	1	1
17. Dilaurylnicotinium dichloride Dark brown paste	10	10	1
18. Dibenzylnicotinium dibromide Red-brown crystals	0	0	1
19. Di-*p*-chlorobenzylnicotinium dichloride Dark brown syrup Insol. residue—10.8 mg./g.	10	5	0
20. 2-(2-Butoxyethoxy)-ethylnicotinium *p*-toluenesulfonate Dark brown syrup	0	0	1
21. 1,2-Ethylene-bis(nicotinium bromide) Dark brown syrup	0	0	1
22. 1-Methyl-3-[2-(1-methylpyrrolidyl-hydriodide)]-pyridinium chloride Orange crystals	0	0	0
23. 1-*p*-Chlorobenzyl-3-[2-(1-methylpyrrolidyl-hydriodide)]-pyridinium chloride Yellow-orange crystals	0	0	0

TABLE II

Bacteriostatic Action of Quaternary Pyridinium Salts on Mycobacterium tuberculosis H37Rv

Quaternary salt with description of compound[3]	Inhibition index Dilution 1:10^4	1:10^5	1:10^6
24. Tetradecylpyridinium chloride White crystals	10	10	1
25. Hexadecylpyridinium chloride 10.56% solution	10	10	6
26. Carbotetradecyloxymethylpyridinium chloride Light yellow gel	10	10	2
27. Carbotetradecyloxymethylpyridinium bromide Yellow syrup	10	5	0
28. Stearyloxymethylpyridinium bromide Brown syrup	10	7	2
29. Tetradecyl bromide quaternary salt of nicotinamide White crystals	10	8	0
30. Tetradecyl-γ-picolinium chloride White crystals	10	10	0
31. Hexadecyl chloride quaternary salt of 3-pyridinecarboxyldiethylamide Tan colored powder	10	10	2
32. 4-Tridecyl-N-allyl-pyridinium bromide	10	10	7
33. Benzyl chloride quaternary salt of 4-pyridinecarboxyldodecylamide White crystals	10	10	1

TABLE III

Bacteriostative Action of Quaternary Quinolinium and Isoquinolinium Salts on Mycobacterium tuberculosis H37Rv

Quanternary salt with description of compound[3]	Inhibition index Dilution 1:10^4	1:10^5	1:10^6
34. 1-Methylquinolinium iodide Orange crystals	2	1	2
35. 1-Dodecylquinolinium *p*-toluenesulfonate White crystals	10	8	3
36. 8-Hydroxy-1-octylquinolinium bromide Yellow crystals	10	9	1

TABLE III (Concluded)

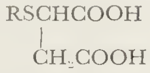
Quanternary salt with description of compound3)	Inhibition index Dilution		
	$1:10^4$	$1:10^5$	$1:10^6$
37. 2-Methylisoquinolinium iodide Yellow crystals	1	1	1
38. 2-Dodecylisoquinolinium *p*-toluenesulfonate White crystals	10	10	3

DISCUSSION

The effect of lengthening alkyl chains on anti-bacterial activity has been investigated with many types of molecules. 4-*n*-Alkylresorcinols in which the alkyl group contained from 6 to 14 carbon atoms inhibited the growth of *M. tuberculosis* H37 in Long's synthetic medium at 10^{-4} per cent but not at 10^{-5} per cent. The resorcinols with alkyl groups of less than 6 carbon atoms were less active (*10*). Using *M. tuberculosis bovis* and *M. smegmatis*, as well as *M. tuberculosis* H37, Barry and coworkers (*11, 12*) reported that for alkylated succinic acids the maximum inhibitory effect *in vitro* was obtained when the compound contained a total of 14 carbon atoms, and that below 12 and above 16 carbon atoms the activity markedly decreased.

For a series of S-*n*-alkylthiomalic acids (**I**) the maximum activity

I.



$$\begin{array}{l} \mathrm{RSCHCOOH} \\ \quad | \\ \quad \mathrm{CH_2COOH} \end{array}$$

was observed when $R=C_{12}$ to C_{14} (*13*). From a study of a series of saturated fatty acids Drea (*2*) reported that maximum inhibition against *M. tuberculosis* H37 was obtained with myristic and palmitic acids. Stearic and lauric acids were less inhibitory, and from C_{12} to C_5 the activity progressively decreased. Using various disubstituted acetic

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3) Sources of compounds:

No. 17—Park, Davis & Company.

Nos. 24, 29–33 inclusive—The Upjohn Company.

No. 25—The William S. Merrell Company.

Nos. 26, 27 and 28—Division of Medicinal Chemistry, The Squibb Institute for Medical Research.

No. 36—The Lilly Research Laboratories.

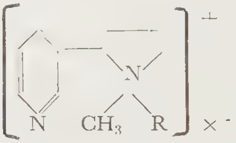
Remainder—Eastern Regional Research Laboratory, U.S. Dept. of Agriculture.

acids over a wide range of molecular weights. Stanley and coworkers (*14*) noted that the activity of this series of compounds reached a maximum when the acids contained a total of 16, 17, and 18 carbon atoms, and those with 19 and 20 carbon atoms were much less active. On the other hand, lengthening the alkyl chain from methyl to dodecyl in polyhalogenated aryl alkyl ethers is reported to abolish the activity against tubercle bacilli when tested *in vivo* using the formation of tubercles on the omentum of infected guinea pigs as criterion of effect (*15*).

In general the bacterial growth inhibitory activity of quaternary ammonium compounds is reported to increase with increasing chain length from C_6 on and reach a maximum at C_{16} (*16*).

In our series of alkylnicotinium halides (**II**) activity in the concentrations tested appeared when R contained 8 carbon atoms. Dodecyl-, hexadecyl-, and octadecyl-nicotinium bromides, and octadecylnicotinium laurate partially inhibited the growth of tubercle bacilli at a dilution of 1:10^5. Didodecylnicotinium dichloride completely inhibited growth at 1:10^5 (see Table I). In the pyridinium series (see Table II) tetradecyl- and hexadecyl-pyridinium chlorides were completely inhibitory at a dilution of 1:10^5. Thus, the alkylnicotinium salts appear to be somewhat less effective than the corresponding simpler alkylpyridinium salts.

II.

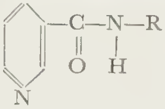


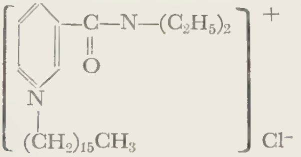
Carbotetradecyloxymethylpyridinium chloride is also completely inhibitory at 1:10^5. It is of interest that the efficacy of the tetradecyl chain is not diminished when it is incorporated into the molecule as a carboalkoxyl group rather than as an alkyl group.

Although the benzyl- and *o*-chlorobenzyl-nicotinium ions showed no significant activity, activity resulted when the chlorine was introduced into the *p*-position of the benzyl nucleus. The introduction of two chloro substitutents in either the 2,3- or the 3,4-positions of the benzyl nucleus appears to increase the activity very little, if any, above that obtained with the *p*-chloro compound. More effective was the addition of a second *p*-chlorobenzyl group to form the diquaternary salt, di-*p*-chlorobenzylnicotinium dichloride. (see Table I).

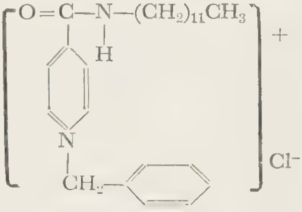
Nicotinamide at a concentration of 200 mg. per 100 ml. has been reported to arrest the growth of tubercle bacilli *in vitro*, and suppress the disease in experimentally infected animals (*17*, *18*). Various amide substituted nicotinamides (**III**) were reported to be less active and or more toxic *in vivo* than nicotinamide itself (*19*). N-Dodecylnicotinamide was reported to be inactive, while N-isopropyl- and N-butylnicotinamides were shown to have about 25 per cent of the activity of streptomycin in experimental mice. In our *in viiro* experiments, we have found the tetradecyl bromide quaternary salt of nicotinamide completely inhibitory at $1:10^4$ and partially so at $1:10^5$, although nicotinamide itself failed to show any growth inhibitory effect at $1:10^4$. Two related salts, the hexadecyl chloride quaternary salt of 3-pyridine carboxyldiethylamide (**IV**) and the benzyl chloride quaternary salt of 4-pyridine-carboxyl dodecylamide (**V**) were both found to be completely inhibitory at $1:10^5$.

III.





IV.



V.

8-Hydroxy-1-octylquinolinium bromide was found to be completely inhibitory at $1:10^4$ and to show some inhibition at $1:10^5$. The presence of the octyl chain in the quaternary ammonium group appears to be without particular significance as far as its activity against the tubercle bacilli is concerned, for 8-hydroxyquinoline sulfate has also been reported to be inhibitory at a dilution of $1:10^5$ in homogenous liquid cultures and in other cultures at $1:6.6 \times 10^4$ to $1:8 \times 10^4$ dilutions (*20*).

Some of the other quaternary salts containing the quinoline or isoquinoline nucleus which were tested also proved to be completely inhibitory at dilutions of $1:10^4$–$1:10^5$ (see Table III).

SUMMARY

A series of pyridinium, nicotinium, quinolinium and isoquinolini-

um quaternary salts were tested for growth inhibiting activity against *Mycobacterium tuberculosis*, strain H37Rv, in Youmans' modification of Proskauer-Beck liquid synthetic medium.

Many of the compounds tested gave complete growth inhibition at a dilution of 1:10^5 but none were completely inhibitory at 1:10^6. The effect of chain length and structure on activity is discussed.

The author gratefully acknowledges the expert guidance of Dr. C. Richard Smith of Barlow Sanatorium under whose direction this investigation was conducted, and of Dr. Milton C. Kloetzel of the Department of Chemistry, University of Southern California and Dr. Cyrus O. Guss, presently of the Department of Chemistry, Colorado Agricultural and Mechanical College, whose advice on the selection of compounds and analysis of data were vital to the successful completion of this work.

The author is also greatly indebted to Drs. D. H. Saunders and L. Weil of the Eastern Regional Research Laboratory, U.S. Department of Agriculture, and to the other suppliers of the chemicals used in this investigation.

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BACTERIOSTATIC EFFECT OF CATIONIC SURFACE ACTIVE AGENTS ON MYCOBACTERIUM TUBERCULOSIS H37R[1]

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The wide spread development and use of surface active substances as cleansing agents have been accompanied by thorough experimental studies on their effect on various microorganisms. Our studies with these substances revealed that even the widely used quaternary ammonium salts were practically useless as disinfectants against the tubercle bacilli (*1*). They had very little " killing " effect on the human tubercle bacilli used in the tests. To further test these compounds, growth inhibition studies on *Mycobacterium tuberculosis* H37Rv were carried out. A report on a study of a series of pyridinium and nicotinium quaternary salts has already been presented for publication (*2*). The present paper is on the remaining group of cationic surface active agents tested in our laboratory.

EXPERIMENTAL

The experimental method has been described previously in detail (*2*). Briefly, 0.1 ml. of a suspension containing 0.1 mg. wet weight of *Mycobacterium tuberculosis* H37Rv was planted into Proskauer-Beck liquid synthetic medium containing the chemicals being tested. The mixture was incubated at 37° for 6 weeks, and visually inspected for bacterial growth at weekly intervals by comparison with controls and blanks. Initial pH and surface tension measurements were made, also, on each detergent-medium mixture.

RESULTS

The results obtained are tabulated in the accompanying tables. S.T. in the tables refers to surface tension in dynes per centimeter, and

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I. I., to inhibition index determined as previously described (*2*). An inhibition index of 10 indicates complete growth inhibition; 0, no inhibition; and intermediate values, partial inhibition.

TABLE I

Bacteriostatic Effect of Quaternary Amine Salts on Mycobacterium tuberculosis H37Rv

Surface active product[2]	Dilution	pH	S.T.	I.I.
1. Hexadecyltrimethylammonium bromide (LAR-222, 17)	$1:10^{4}$	7.3	36	10
	$1:10^{5}$	7.3	41	10
	$1:10^{5.3}$	7.3	54	10
	$1:10^{5.6}$	7.5	57	10
	$1:10^{5.9}$	7.3	53	3
	$1:10^{6}$	7.3	58	0
	$1:10^{6.2}$	7.3	59	0
2. Hexadecyltrimethylammonium bromide (Ctab, 3; Cetamium, 11)	$1:10^{4}$	6.9	34	10
	$1:10^{5}$	6.9	43	10
	$1:10^{6}$	6.9	62	0
3. 9-Octadecenyldimethylethylammonium bromide (Thoral, 17)	$1:10^{4}$	6.9	35	10
	$1:10^{5}$	6.9	36	10
	$1:10^{6}$	6.9	49	1
4. Dialkyldimethylammonium chloride, average mol. wt.=523, made with fatty acids from coco (Arquad 2C, 2)	$1:10^{4}$	7.2	28	10
	$1:10^{5}$	7.3	36	10
	$1:10^{6}$	6.9	52	3
5. Dialkyldimethylammonium chloride, average mol. wt.=608, made with tallow fatty acids (Arquad 2HT, 2)	$1:10^{4}$	7.3	31	10
	$1:10^{5}$	7.1	52	3
	$1:10^{6}$	7.3	54	1
6. Alkyltriethylammonium sodium sulfate, alkyl =16 & 18 C (A-509-R, 7)	$1:10^{4}$	7.0	33	10
	$1:10^{5}$	7.1	43	10
	$1:10^{6}$	7.2	57	0
7. Alkyldimethylbenzylammonium chloride, alkyl =8 to 18 C (LAR-257, 17)	$1:10^{4}$	7.0	31	10
	$1:10^{5}$	6.9	41	10
	$1:10^{6}$	7.1	54	0
8. Alkyldimethylbenzylammonium chloride, alkyl =8 to 18 C (Zephiran, 21)	$1:10^{4}$	6.9	31	10
	$1:10^{5}$	6.8	43	10
	$1:10^{6}$	6.6	49	4
9. Alkyldimethyl-3,4-dichlorobenzylammonium chloride (LAR-222, 17)	$1:10^{5}$	7.0	53	10
	$1:10^{5.3}$	7.0	53	10
	$1:10^{5.6}$	7.0	56	10
	$1:10^{5.9}$	7.0	61	0
	$1:10^{6.2}$	7.0	56	0
10. Benzylphenyldimethylammonium chloride (01420 Lot R11–1446, 10)	$1:10^{4}$	6.9	62	0
	$1:10^{5}$	6.9	63	0
	$1:10^{6}$	6.9	62	0

TABLE I (continued)

Surface active product[2]	Dilution	pH	S.T.	I.I.
11. (1-Benzyldodecyl)-benzyldimethylammonium chloride (OC-160-2842-II, 15)	$1:10^4$	7.1	35	10
	$1:10^5$	7.2	37	10
	$1:10^6$	6.9	46	10
	$1:10^6$	7.2	46	9
	$1:10^7$	7.0	51	2
	$1:10^8$	7.1	59	2
12. (1-Phenyldodecyl)-dimethylcinnamylammonium chloride (OC-160-2829-IV, 15)	$1:10^4$	7.0	33	10
	$1:10^5$	7.2	33	10
	$1:10^6$	7.2	59	9
13. Hexadecyldiethyl-(β-hydroxyethyl)--ammonium chloride (238, 12)	$1:10^4$	6.8	35	10
	$1:10^5$	6.9	45	10
	$1:10^6$	6.6	50	3
14. Hexadecyldi-*n*-propyl-(β-hydroxyethyl)-ammonium chloride (260,12)	$1:10^4$	6.8	36	10
	$1:10^5$	6.9	37	10
	$1:10^6$	6.9	62	6
15. β-(Dibenyzlamino)-ethanol methiodide (01403 Lot W2-10-9-6, 10)	$1:10^4$	7.1	33	8
	$1:10^5$	6.9	42	0
	$1:10^6$	7.2	59	0
16. Dimethyl-2-(2-hydroxyethoxy)-ethyloctadecylammonium chloride (03430, 10)	$1:10^4$	6.9	42	10
	$1:10^5$	6.9	44	9
	$1:10^6$	7.0	53	3
17. (*p*-tert.-Octylphenoxyethoxyethyl)-trimethylammonium chloride (Ditto, 13)	$1:10^4$	7.1	32	10
	$1:10^5$	6.8	45	3
	$1:10^6$	6.9	44	3
18. (*p*-Diisobutylphenoxyethoxyethyl)-dimethylbenzylammonium chloride (Hyamine 1622, 14)	$1:10^4$	6.9	35	10
	$1:10^5$	6.9	39	10
	$1:10^6$	6.9	50	0
19. (2,2,4,4-Tetramethylbutylphenoxyethoxyethyl)-dimethylbenzylammonium chloride (*, 22)	$1:10^4$	8.3	32	10
	$1:10^5$	6.9	37	10
	$1:10^6$	6.8	43	4
20. (*p*-tert.-Octylphenoxyethoxyethyl)-dimethylbenzylammonium chloride (Teramine, 20)	$1:10^4$	7.1	37	10
	$1:10^5$	7.2	42	10
	$1:10^6$	7.1	44	9
21. (*p*-tert.-Ocytylphenoxyethoxyethyl)-dimethylcinnamylammonium chloride (Ditto, 13)	$1:10^4$	7.3	32	10
	$1:10^5$	7.0	40	10
	$1:10^6$	7.1	54	6
22. Trimethyl-(3-dodecyloxymethyl-4-methylphenyl)-ammonium sulfate (Ditto, 9)	$1:10^4$	6.8	34	10
	$1:10^5$	7.0	36	10
	$1:10^6$	6.9	48	4
23. Diethylaminoethyloleylamide methosulfate (Sapamine MS, 5)	$1:10^4$	7.0	35	10
	$1:10^5$	6.9	41	10
	$1:10^6$	6.8	55	0
24. Phenyldimethylaminoethylketone methosulfate (01426 Lot 2R11-1346, 10)	$1:10^4$	6.9	54	10
	$1:10^5$	6.8	54	0
	$1:10^6$	6.8	54	0

TABLE I (concluded)

Surface active product[2]	Dilution	pH	S.T.	I.I.
25. β-Benzhydryloxyethyldimethyl-(carbo-2-ethylhexyloxymethyl)-ammonium chloride (BS-8, 13)	$1:10^4$	6.8	37	10
	$1:10^5$	6.9	42	7
	$1:10^6$	6.9	62	0
26. Benzyldiethyl-(heptadecylcarbamylethyl)-ammonium bromide (MC-473, 16)	$1:10^4$	6.6	34	10
	$1:10^5$	6.8	38	10
	$1:10^6$	6.8	50	10
	$1:10^6$	7.0	50	9
	$1:10^7$	7.2	62	2
	$1:10^8$	7.3	62	0
27. Benzyldiethyl-2-(heptadecylcarbamyl)-ethylammonium chloride (MC-467, 16)	$1:10^4$	7.0	31	10
	$1:10^5$	7.1	48	10
	$1:10^6$	7.0	55	5
28. Tris-(2-hydroxyethyl)-2-(octadecylcarbamyl)-ethylammonium chloride (04487, 10)	$1:10^4$	7.0	47	10
	$1:10^5$	7.0	53	5
	$1:10^6$	7.0	59	0
29. N-(Acylcolaminoformylmethyl)-pyridinium chloride (Emulsept, 8)	$1:10^4$	6.9	28	10
	$1:10^5$	6.9	41	9
	$1:10^6$	6.9	56	0
30. 2-*p*-Diethylaminostyryl-6-methylquinoline ethochloride (Rx-K 125-2, 13)	$1:10^4$	6.4	42	10
	$1:10^5$	6.9	46	10
	$1:10^6$	6.8	54	4
31. Laurylisoquinolinium bromide (LAR-262, 17)	$1:10^4$	7.0	36	10
	$1:10^5$	7.2	41	10
	$1:10^6$	7.2	49	7
32. 1-Laurylbenzotriazole ethobromide (KIII-284, 18)	$1:10^4$	7.2	38	10
	$1:10^5$	7.0	34	10
	$1:10^6$	6.6	47	4
33. N-Tetradecylbenztriazole ethobromide (KIII-286, 18)	$1:10^4$	7.1	38	10
	$1:10^5$	7.1	35	10
	$1:10^6$	7.0	44	6
34. (*p*-tert.-Octylphenoxyethoxyethyl)-morpholine hydrochloride (BS-6, 13)	$1:10^4$	6.8	33	10
	$1:10^5$	7.0	35	9
	$1:10^6$	7.1	57	2
35. Ethyl-*n*-hexadecyl-2-ketomorpholinium bromide (BS-7, 13)	$1:10^4$	6.8	32	10
	$1:10^5$	6.7	40	5
	$1:10^6$	6.8	61	2
36. Hexadecylpyridinium sulfathiazole (Cepryn sulfathiazole, 19)	$1:10^4$	7.1	35	10
	$1:10^5$	7.3	53	0
	$1:10^6$	7.4	61	0
37. Hexadecyltrimethylammonium sulfathiazole (Cetamium sulfathiazole, 19)	$1:10^4$	7.2	35	10
	$1:10^5$	7.0	61	0
	$1:10^6$	7.0	65	0
38. Hexadecylpyridinium sulfapyridine (Cepryn sulfapyridine, 19)	$1:10^4$	6.8	32	10
	$1:10^5$	7.0	49	10
	$1:10^6$	7.0	59	0

TABLE II

Bacteriostatic Effect of Alkyl Amines on Mycobacterium tuberculosis H37Rv

Surface active product[2)]	Dilution	pH	S.T.	I.I.
39. Tetradecylamine (Armeen 14, 2)	$1:10^4$	7.0	42	10
	$1:10^5$	7.0	57	10
	$1:10^6$	6.9	57	4
40. Octadecylamine (Armeen 18 D, 2)	$1:10^4$	7.0	58	6
	$1:10^5$	6.9	52	3
	$1:10^6$	6.9	57	5
41. 1-Hydroxyethyl-2-heptadecenylimidazoline (Amine 0, 1)	$1:10^4$	7.0	32	10
	$1:10^5$	7.0	38	9
	$1:10^6$	7.1	45	0
42. 1-Hydroxyethyl-2-heptadecenylimidazolinium chloride (Quaternary 0, 1)	$1:10^4$	7.5	31	10
	$1:10^5$	7.3	47	9
	$1:10^6$	7.2	59	0
43. Heptadecylbenzinimidazole monosulfonate (Ultravon K, 5)	$1:10^4$	7.3	47	10
	$1:10^5$	7.3	59	0
	$1:10^6$	7.2	59	0
44. Morpholine (Ditto, 4)	$1:10^4$	7.0	62	0
	$1:10^5$	6.8	67	0
	$1:10^6$	6.9	53	0

TABLE III

Bacteriostatic Effect of Thiouronium Salts on Mycobacterium tuberculosis H37Rv

Surface active product[2)]	Dilution	pH	S.T.	I.I.
45. Triglycoldithiouronium dihydrochloride (BS-1, 13)	$1:10^4$	6.9	56	0
	$1:10^5$	6.9	54	0
	$1:10^6$	6.9	64	0
46. Benzhydrylisothiourea hydrobromide (BS-3, 13)	$1:10^4$	6.7	52	0
	$1:10^5$	6.7	54	0
	$1:10^6$	6.4	51	0
47. *p*-tert.-Octylphenoxyethoxyethylisothiourea hydrochloride (BS-5, 13)	$1:10^4$	7.0	35	10
	$1:10^5$	6.9	39	10
	$1:10^6$	6.9	60	0

TABLE IV

Bacteriostatic Effect of Miscellaneous Cationic Compounds on Mycobacterium tuberculosis H37Rv

Surface active product[2)]	Dilution	pH	S.T.	I.I.
48. Dimethyldodecylamine oxide (RO-D, 21)	1:10^4	7.0	29	10
	1:10^5	6.9	40	10
	1:10^6	7.0	57	0
49. Tris-(hydroxymethyl)-amino methane (Ditto, 6)	1:10^4	6.9	49	0
	1:10^5	6.8	65	0
	1:10^6	6.9	61	0

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2) Some of the products are pure compounds; others are mixtures. The latter are listed under what is presumed to be the main active ingredient for each. Their detailed compositions are described in a previous publication (*1*). The dilutions listed are dilutions of the surface active agents.

In parenthesis after each compound name are given the trade name or the supplier's designation of the product and a number referring to the source of the product as follows:

1. Alrose Chemical Company
2. Armour and Company
3. J. T. Baker Chemical Company
4. Carbide and Carbon Chemicals Corporation
5. Ciba Company
6. Commercial Solvents Corporation
7. Emery Industries, Incorporated
8. The Emulsol Corporation
9. Givaudan-Delawanna, Incorporated
10. The Lilly Research Laboratories
11. The William S. Merrell Company
12. Ortho Research Foundation
13. Parke, Davis & Company
14. Rohm & Haas Company
15. Sharpe & Dohme, Incorporated
16. Division of Medicinal Chemistry, The Squibb Institute for Medical Research
17. Turco Products, Incorporated
18. The Upjohn Company
19. Vick Chemical Company
20. West Disinfecting Company
21. Winthrop-Stearns, Incorporated
22. Wyandotte Chemicals Corporation

* Trade name was omitted at the request of the supplier.

DISCUSSION

Among the early studies on the effects of quaternary ammonium compounds is a report by Domagk (*3*) who indicated that the tubercle bacilli were unaffected by practical dilutions of alkyldimethylbenzylammonium chloride. Using a non-virulent Novy strain of tubercle bacilli Freedlander (*4*) reported that Zephiran, and alkyldimethylbenzylammonium chloride, completely inhibited growth at 1:80,000 dilution in a liquid synthetic medium, and partially at as high a dilution as 1:400,000. Drea (*5*) noted that in Long's liquid synthetic medium Zephiran permitted the growth of *M. tuberculosis* H37 at a dilution of 10^{-5} per cent, but prevented growth at 10^{-4} per cent. Under our experimental conditions Zephiran (No. 8) gave results intermediate between those of Freedlander and Drea.

Phemerol, described as a *p*-tertiary-octylphenyldiethoxydimethylbenzylammonium chloride, which is probably very similar, if not identical with our Nos. 18, 19, and 20, was reported to prevent growth at 10^{-3} per cent but not at 10^{-4} per cent (*5*). This is in the range of our results, also.

An adequate discussion of the relation of structure to activity is somewhat hindered by the fact that many of the substances tested are commercial products whose exact chemistries are not known. However, certain trends can be noted. Most of the quaternary amine compounds had some growth inhibitory effect on *Mycobacterium tuberculosis* H37Rv at a dilution of $1:10^5$, and all except two were completely inhibitory at $1:10^4$ dilution (see Table I). The two exceptions, Nos. 10 and 15, both lack long alkyl chains confirming an often observed fact that the length of an alkyl chain is an important factor in the antibacterial effect of many types of compounds (*6*). No. 15 with somewhat longer substituents was more effective than No. 10. The benzyl group, present in both No. 10 and No. 15, as well as in several others with long alkyl chains, appears to add very little, if any, antibacterial activity to the quaternary ions. Chloride substitutions in the benzene ring as in No. 9 seems to offer no advantages as compared to similar non-chlorinated compounds Nos. 7 and 8.

Morpholine was reported to permit growth at 10^{-1} and 1 per cent dilutions (*5*). We, also, found no inhibition of the tubercle bacilli by this compound at $1:10^4$ dilution (see Table II). Two long chained derivatives of morpholine, Nos. 34 and 35, were more effective, but not quite as effective as similar simpler ammonium derivatives (see Table I).

The combination of a quaternary ion with a sulfa group had no additive nor synergistic effect (Nos. 36, 37, and 38). Rather, the results indicate depression of activity. Cetylpyridinium chloride, itself, is reported by Drea (*5*) to prevent growth of *M. tuberculosis* H37 at 10^{-4} per cent while permitting growth at 10^{-5} per cent. Our results with this compound gave complete inhibition at $1:10^5$ dilution and partial inhibition at $1:10^6$ dilution (*2*). Cetamium (No. 2, also No. 1) gave complete inhibition at $1:10^5$ dilution, also. The endpoint of activity for sulfathiazole on the tubercle bacilli was reported to be around 10^{-3} per cent by Drea (*5*), while Muller (*7*) found this compound to be bacteriostatic at 5 mg. per ml. Sulfapyridine, reported to give complete growth inhibition of the tubercle bacilli at 1:1000 and partical inhibition at 1:5000 (*8*) and at 80 mg. per cent (*9*), is less active than sulfathiazole (*10*, *11*), but had a less depressing action on the activity of the quaternary, Cepryn.

Most active of the alkylamines tested was a product designated as Armeen 14 (No. 39), and reported by the supplier to be 90 per cent tetradecyl-, 4 per cent dodecyl-, 4 per cent hexadecyl-, and 2 per cent octadecenyl-amines. Armeen 18D, 93 per cent octadecyl-, 6 per cent hexadecyl-, and 1 per cent octadecenyl-amines, was found to be much less active. The predominantly octadecylamine product was found to be less active in bactericidal tests against the tubercle bacilli, also (*1*).

Under our experimental conditions the thiourea group does not appear to contribute any significant activity to the molecule. *p*-Tertiary octylphenoxyethoxyethylisothiourea hydrochloride, the only one of the three compounds tested in this group which showed activity, was found to be no more active than the quaternary amines with the octylphenoxyethoxyethyl chain.

Concerning the effect of surface tension, Alexander and Soltys (*12*) reported that growth of *Mycobacterium tuberculosis*, human type designated as "DT," was inhibited at surface tensions below about 42 dynes per centimeter. Our observations with the cationic detergents are generally in accord with their results. No growth, or only partial growth occurred at surface tensions below about 40–45 dynes per centimeter with one exception (No. 15). However, the growth inhibiting effect of these substances cannot be attributed wholly to their effect on surface tension, for complete growth inhibitions have been observed at surface tensions of around 55 dynes per centimeter with some of the tested products.

SUMMARY

A group of 49 cationic surface active agents were tested for bacteriostatic activity against *Mycobacterium tuberculosis* H37Rv. Many of the substances tested were completely inhibitory at a dilution of 1:10^5, and a few were highly inhibitory at 1:10^6 dilution. Only partial growth, if any, was observed at surface tensions of less than about 40–45 dynes per centimeter, although complete inhibitions were obtained with some of the tested substances at as high a surface tensions as 54–57 dynes per centimeter.

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ENZYMATIC HYDROLYSIS OF THIOESTERS, ESFECIALLY IN RELATION TO O-ESTERASE

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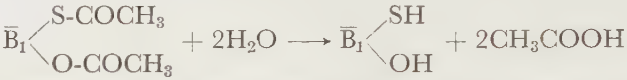
One of the physiological meanings of -SH groups in living matters has been considered to be its -SH $\rightleftarrows$ -SS- redox-system, but there has been found no experimental evidence for the notion that the system plays an important role in the main hydrogen-transporting pathways. On the other hand, the system is well recognized as the rH-regulating system, which offers the most favourable field to extensive cellular functions, being similar to the buffering mechanism to maintain the H_3O^+ concentration at the optimal conditions. Recently, there has been reported another and remarkable role of -SH groups. Namely, it forms -S-CO- linkage with several substrates in their enzyme-substrate complexes. For examples, Racker (*1*) and Crook *et al* (*2*) showed that -S-CO- is formed between cofactor, glutathione, and glyoxal in glyoxalase action, and Racker *et al* (*3*) expanded these findings to 3-phosphotriose dehydrogenase. On the other hand, Lynen clarified the nature of the " active C_2-compound " in the enzymatic transacetylation by demonstrating the formation of acetyl coenzyme A (*4*). More recently, Reed and DeBusk (*5*) investigated the mechanism of the oxidative decarboxylation of pyruvic acid to propose the intermediary formation of acetyl lipothiamide combined with the oxidized substrate by the -S-CO- bond. The significance of -SH radicals in several enzymatic activities have been widely emphasized on the phenomenal basis, whose experimental evidences depended mainly on the fact that the -SH inhibitors greatly inhibit the, so called, -SH enzymes. But now the above-mentioned investigations demonstrate one of the roles of -SH groups in the enzymatic reactions on the substantive foundation that many enzyme-substrate complexes are combined structurely by -S-CO- linkage.

Many investigations carried out in our country on the mode of action of thiamine, have been given attention to the redox-system.

$$2\ \bar{B}_1\text{-SH} \rightleftarrows \bar{B}_1\text{-S-S-}\bar{B}_1$$

In such a manner, "allithiamine," one of the disulfide derivatives of thiamine, was found in the mixture of homogenized garlic with thiamine (*6*) and it was further studied on its isolation and chemical structure by Matsukawa and Yurugi (*7*). On the contrary, most of the foreign investigators have payed their attentions chiefly to 4-amino group of pyrimidine ring in thiamine as the active group (*8*, *9*) since the study of Langenbeck (*10*) on his carboxylase-model. In such a situation it is noticeable that Matsukawa and Kawasaki (*11*) synthesized a new derivative of thiamine, O, S-diacetyl thiamine (DAT). During our biochemical studies on it, we found that it is readily hydrolyzable into thiamine and acetic acid by liver extracts (*12*).



$$\bar{B}_1\langle\begin{matrix}\text{S-COCH}_3\\ \text{O-COCH}_3\end{matrix} + 2H_2O \longrightarrow \bar{B}_1\langle\begin{matrix}\text{SH}\\ \text{OH}\end{matrix} + 2CH_3COOH$$

Concerning the enzymatic hydrolysis of -S-CO- linkage, there has been found no report except "succinyl- and acetyl coenzyme A deacylases" investigated by Gergeley *et al* (*13*), although a great many of studies have been carried out on various esterases capable to hydrolyze ordinary ester linkages (abbreviated -O-CO- ester as "O-ester"). Then, we studied on the enzymatic hydrolysis of the lower thioesters (abbreviated thioester as "S-ester"), such as methyl thioacetate (MeSAc) and ethyl thioacetate (EtSAc) by liver enzymes, comparing with that of methyl acetate (MeOAc) and ethyl acetate (EtOAc). The results are described below.

METHODS

Substrates—MeSAc and EtSAc were synthesized according to the method of Obermeyer (*14*). Acetyl thiocholine was kindly offered by Prof. T. Suzuki of Kyoto University.

The enzyme assays—We used the following three methods in order to measure the esterase-activity.

i) Manometric method: We estimated the esterase activity by measuring manometrically the CO_2-HCO_3^- buffer system (*15*) in most of the following experiments.

ii) Application of the hydroxamic acid reaction to the determination of S-esters: We applied the alkali-NH_2OH method of Hestrin (*16*) to determination of MeSAc and EtSAc and found that both formed ferric complex of acethydroxamic acid, whose $\lambda_{max.}$ was found at 510 mμ and therefore they are quantitatively determinable by measuring the extinction coefficients at 510 mμ by means of Beckman spectrophotometer, Model B, as shown in Fig. 1.

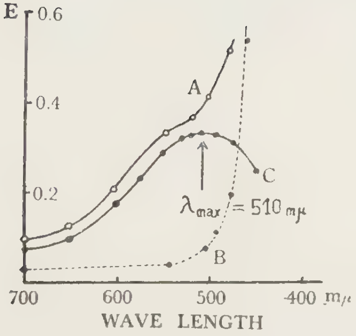


Fig. 1. Absorption by ferric acethydroxamic acid complex formed from 2μM of ethyl thioacetate in 5 ml. of final solution.

A, total extinction; B, reagent blank; C, absorption by ferric acethydroxamic acid complex (calculated by difference).

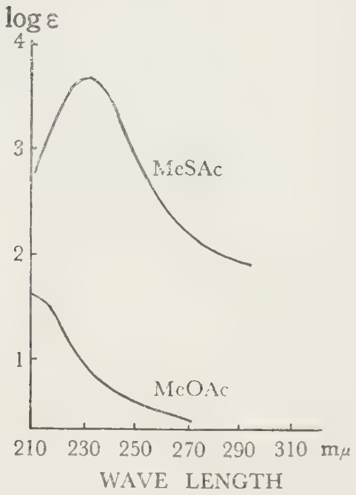


Fig. 2. Absorptoin spectra of methyl thioacetate.

iii) Differential determination of S-ester from O-ester by ultraviolet absorption: One can determine S-ester differentially from O-ester neither by manometry nor the hydroxamic acid reaction. But we found that S-ester has absorption maximum at 230 mμ, where there is almost no absorption by O-ester (Fig. 2). Accordingly, one can determine S-ester solely without the influence of the coexistence of O-ester.

RESULTS

Distribution of S-esterase Activity in Rat Tissues

The tissues distribution of S-esterase was firstly investigated, comparing with that of O-esterase activities. As shown in Table I, livers were by far the most active tissues, while heart muscle, brain, spleen and skeletal muscle contained very little of S-esterase. It is noticeable that S-esters are much more hydrolyzable than O-esters and that the tissue distribution of S-esterase is quite accordant with that of O-esterase.

Then, we studied on the hydrolysis of acetyl choline and acetyl

TABLE I

Distribution of S- and O-esterase Activities in Rat Tissues

Tissue*	Activity (cu.mm./10 min·)**			
	Methyl acetate	Methyl thioacetate	Acetyl choline	Acetyl thiocholine
Liver	113	679	2	3
Kidney	8	179	—	—
Spleen	1	17	—	—
Heart muscle	0	11	—	—
Brain	0	17	64	58
Skeletal muscle	0	7	—	—
Blood (rabbit)	14	34	—	—

* Each tissues were homogenized with 20 volumes of water and after centrifuging the resulting extracts were used as enzyme solutions.

** Final concentrations of each substrates: Methyl acetate, methyl thioacetate, 0.02 *M*; acetyl choline, 0.0025 *M*; acetyl thiocholine, 0.002 *M*.

thiocholine as substrate and found that brain showed active hydrolyzing activities on both substrates, while liver did not and that unlike in case of MeSAc and MeOAc, acetyl thiocholine was as hydrolyzable as acetyl choline (Table I).

Preparation of the -S-CO- Esterolytic Enzyme

For the purpose of separating the MeSAc-hydrolyzing enzyme differentially from O-esterase, we intended the purification. The blood-free liver was taken out from the rabbit perfused with about 500 ml. Ringer solution and homogenized with 20 volumes of water. After centrifuging the extract was added with 3 volumes of cold acetone and the resulting precipitate was dried in vaccuum desicator. Extracts of the acetone powder was made by adding 1 per cent glycerol solution and centrifuging. The supernatants were fractionated by salting out with ammonium sulphate. The fraction obtained between 0.4 and 0.6 saturations with ammonium sulphate contained most of both activities. The precipitate was then lyophilized and then it was stably conservable at least for a month. 100 mg. of the powder was extracted with 10 ml. of water with vigorous shaking. The resulting supernatant was almost colerless and transparent after centrifuging and was used in much of the subsequent work. In case of the acetone- or ammonium sulphate-fractionation of the enzymes from liver extracts, S-esterase distributed in parallel with O-esterase in every fractions. Table II indicates one of such results. In conclusion, the S-esterase activity could not be separated from the O-esterase activity.

TABLE II

Distribution of O- and S-ester Hydrolyzing Activities in Each Fractions Obtained by Ammonium Sulphate Fractionation of Liver Extract

Fraction	Activity (cu.mm./10 min.)	
	Methyl acetate	Methyl thioacetate
Saturation with ammonium sulphate		
0 ——0.2	7.8	54
0.2——0.5	21.4	213
0.5——0.75	3.6	119

Comparisons of Several Enzymatic Properties of both S- and O-esterase Activities of the Liver Enzyme

Comparing with the hydrolysis of MeOAc, we studied on several properties of the enzymatic hydrolysis of MeSAc by the liver enzyme.

pS-activity—The relation between substrate concentration and both hydrolyzing activities are shown in Fig. 3. MeSAc and EtSAc are 7–8 times more rapidly hydrolyzable than MeOAc and EtOAc. The respective Michaelis constants (Km) are also indicated.

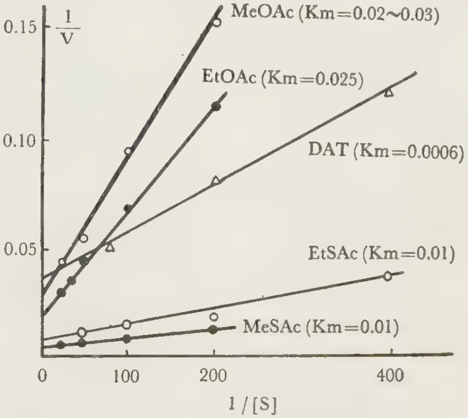


FIG. 3. pS-Activity relationships for the enzymatic hydrolyses of several O-esters and S-esters by the liver enzyme.

On the other hand, the results on the hydrolysis of acetyl choline and acetyl thiocholine by brain homogenates are demonstrated in Table III. Unlike the hydrolysis of the lower simple O- and S-esters by the liver enzyme, acetyl thiocholine was hydrolyzed

TABLE III

The pS-activity Relationships in the Hydrolysis of Acetyl Choline and Acetyl Thiocholine by Brain Homogenates

Acetyl choline		Acetyl thiocholine	
Concentration	Activity	Concentration	Activity
M	*(cu.mm./5 min.)*	*M*	*(cu.mm./7 min.)*
0.02	12.1	0.032	18.7
0.01	14.5	0.016	22.3
0.0025	16.0	0.004	21.6
0.00125	19.3	0.002	20.6

almost at the same velocity as acetyl choline. The higher concentrations of acetyl thiocholine inhibited its own hydrolysis, as in the case of acetyl choline reported by Augustinsson (*17*).

pH-activity—The optimal pH for the activities of the liver enzyme on EtOAc and MeSAc was determined by the hydroxamic acid reaction in the presence of phosphate or borate-carbonate buffers. These esters, especially S-esters, spontaneously decomposed at the alkaline side of pH, and therefore the corrected values are shown in Table IV. The

TABLE IV

Effect of pH on S- and O-esterase Activities of the Liver Enzyme

pH	Spontanous hydrolysis*		Enzymatic hydrolysis*,**	
	Ethyl acetate	Methyl thioacetate	Ethyl acetate	Methyl thioacetate
6	—	—	0.070	—
7	—	—	0.090	0.061
8	—	0.000	0.140	0.106
8.5	—	—	0.158	0.115
9	—	0.026	0.227	0.066
9.5	0.000	0.031	0.210	0.052
10	0.006	0.036	0.186	0.016
11	0.080	0.080	0.077	0.000

* The relative values of hydrolyzed substrates, indicated by readings of Beckman spectrophotometer according to the hydroxamic acid reaction.

** Corrected values, subtracted with the respective amounts of substrates hydrolyzed spontaneously.

data shows that pH 8.5 was optimal for MeSAc and 9.0 for EtOAc and that both pH-activity relationships coincided well with each other. Although the optimal pH were found between 8.5 and 9.0, we carried out the subsequent works at pH 7.6 in order to avoid perfectly the spontaneous hydrolysis of the substrates.

Heat Stability—Table V shows the stabilities of the liver enzyme on both MeOAc and MeSAc as influenced by temperature. Both activities were inactivated above 60°. It is noticeable that the temperature-stability relationship with MeSAc is also well in accordance with that of MeOAc.

TABLE V

Influence of Temperature on Stabilities of the Liver Enzyme

Temperature*	Relative activities	
	Methyl acetate	Methyl thioacetate
°C		
30	100	100
40	91	88
50	51	68
60	3	19
70	4	3

* Incubated for 10 minutes at the respective temperature.

Temperature-activity—We studied then the influences of temperature on both enzyme activities, as shown in Table VI. The apparent acti-

TABLE VI

Effect of Temperature on the Activity of the Liver Enzyme

Temperature	Activity (*cu.mm./10 min.*)*	
	Methyl acetate	Methyl thioacetate
°C		
18	14.5	33.6
25.5	31.0	50.6
31	39.0	72.7
37	56.6	83.2
45	61.1	92.7

* Determined manometrically.

vation energies of both hydrolyses of MeOAc and MeSAc by the liver enzyme are calculated according to "Arrhenius' equation" to be 10,400 and 5,800 cal., respectively.

Influences of the Inhibitors—It is well-known that liver esterase is sensitive to atoxyl and resistant to quinine, the inhibitor of pancreatic lipase. Table VII indicates that 0.0001 *M* atoxyl inhibits the hydrolysis of MeSAc by the liver enzyme, while quinine inhibit a little even at the higher concentrations (0.01 *M*). The influences of these inhibitors upon S-esterase perfectly paralleled with that upon O-esterase.

TABLE VII

The Effect of Atoxyl and Quinine on the Hydrolyses of Methyl Acetate, methyl Thioacetate and O, S-diacetyl Thiamine by the Liver enzyme

Inhibitor	Final concentration	Inhibition per cent		
		MeSAc	EtOAc	DAT
	M	%	%	%
Atoxyl	0.001	90	92	65
	0.0001	89	86	49
	0.00002	74	73	—
	0.00001	48	55	33
	0.000001	4	6	8
Quinine	0.005	14	7	—
	0.0025	7	0	—
MeOAc	0.1	8	—	47
	0.01	0	—	11

Final concentration of substrate: MeSAc and EtOAc, 0.01 *M*; DAT, 0.008 *M*.

DISCUSSIONS

Is Lower Thioesters Hydrolyzable by Liver O-esterase or a -S-CO- Specific Hydrolase?

As mentioned above, it was clarifed that such lower S-esters as MeSAc and EtSAc are much more hydrolyzable than O-esters by our liver enzyme. Then, the following question arises, whether S-ester is attacked by the liver O-esterase itself or by a S-ester specific hydrolase, which may be called "thioesterase" or "S-esterase." Both S- and

O-esterase activities paralleled well each other in various points. Namely, we can point out, firstly, the accordance of tissue distributions of both esterolytic activities, secondly, the close coincidence of their distributions in every fractions obtained by ammonium sulphate fractionation of liver extracts, and thirdly, parallelism of several properties of both activities, that is, in pH-activity, heat-stability and effect of inhibitors. Judging from these results, we could find no experimental evidence for the occurrence of a -S-CO- specific esterase in the liver. If O-esterase itself attacks not only O-esters by S-esters, the hydrolysis of MeSAc might be inhibited competitively by O-ester. Although we observed by means of ultraviolet absorption method that 1 *M* EtOAc rather inhibited the hydrolysis of MeSAc, we could not ascertain competitive relationships between MeSAc and EtOAc. Accordingly, it is concluded that liver O-esterase itself is able to hydrolyze not only O-esters but also S-esters, possibly due to its loose substrate-specificity. The question, however, remains unsolved, because we could obtain nothing but the negative evidences.

Is the -S-CO- Linkage Able to be Physphorolyzed?

Recently, Lipmann (*18*) and many other investigators demonstrated that coenzyme A is concerned not only in the transacetylation but also in the generation of ATP and that the -S-CO- linkage in acetyl coenzyme A is energy-rich. On the other hand, Racker (*3*) suggested that in the process of the phosphoroclastic oxidation of 3-phosphotriose the phosphorolysis of the -S-CO- containing substrate-enzyme intermediate resulted in the formation of 1,3-diphosphoglyceric acid. Judging from our studies, S-ester is found to be much more hydrolyzable than O-ester, and the activation energy of MeSAc hydrolysis is about two times smaller than that of EtOAc. These facts, indicating the more unstability of -S-CO- than -O-CO- linkage, suggested that the former is energy-rich. Therefore, the following possibility is anticipated that MeSAc might give energy-rich phosphate compound in consequence of the phosphorolysis.

$$R_1\text{-S-CO-}R_2 + \text{HO-PO}_3H_2 \longrightarrow R_1\text{-SH} + R_2\text{CO-O-PO}_3H_2$$

For the purpose of ascertaining such possibility, the following experiments were carried out, but we obtained negative results. Namely, i) there occurred no decrease in inorganic phosphate with MeSAc and our liver enzyme in the presence of phosphate buffer. ii) Even when the esterase activity was perfectly inhibited by 0.0001 *M* atoxyl, we could observe no consumption of inorganic phosphate. iii) Although the hydrolysis of MeSAc by our liver enzyme was somewhat reduced by the presence of phosphate, the inhibition was not reversed by arsenate. These facts show that MeSAc is not phosphorolyzed by our enzyme preparation, which was not conclusively proved as yet, for our enzyme preparation is not sufficiently purified.

SUMMARY

1. The occurrence of an enzyme in mammalian liver was ascertained which was capable to hydrolyze lower thioesters.

2. Of various tissues of rats, the liver was found to have the most active enzymatic activity, while the other tissues exerted almost no activity except kidney and blood. On the other hand, acetyl thiocholine was hydrolyzable not by livers, but well by brain-homogenates.

3. From rabbit livers, we obtained the active enzyme preparation by ammonium sulphate fractionation. Although it contained also O-esterase activity, it attacked thioesters much more rapidly than O-esters.

4. Comparing with O-esterase activity, we investigated several enzymatic properties of S-ester hydrolyzing activity of the liver enzyme, that is, pH-activity, pS-activity, temperature-activity, heat-stability and the influences of atoxyl and other inhibitors. These properties of S-esterase activity are well accordant with those of O-esterase activity.

5. On the basis of these results, there seemed not to exist S-ester specific hydrolyzing enzyme, but O-esterase itself hydrolyzes thioester possibly due to its loose substrate-specificity.

6. We could obtain no experimental evidence for phosphorolysis of these S-esters by our liver enzyme.

RESUMO

1. En hepato de mambesto sin trovis la enzimo kapabla hidrolizi malaltan S-esteron.

2. El diversaj organoj de rato, hepato enhavas la plej fortan enzimagon, dum la aliaj organoj testitaj enhavas neniun aktivecon escepte de reno kaj sango. Aliflanke, acetilsulfĥolino, kiel same al acetilĥolino, estas ne hidrolizebla per hepato, sed per cerbo-homogenito.

3. El hepato de kuniklo ni ekstraktis la enzimon, kiu hidrolizis S-esteron.Kvankam tiu ĉi enhavas ankaŭ O-esterazo-aktivecon, ĝi atakis S-esteron multe pli rapide ol O-esteron.

4. Kompare kun hidrolizo de O-esteroj, ni klarigis kelkajn enzimajn karakterojn

de hidrolizo de S-esteroj pere de nia hepato-enzimo, nome, pH-aktivecon, pS-aktivecon, temperaturo-aktivecon, varmo-stabilecon kaj influon de atoksilo kaj aliaj malhelpantoj. Tiuj ĉi proprecoj de S-esterazo-aktiveco bone koincidis kun tiuj de O-esterazo-aktiveco.

5. Sur la bazo de tiuj ĉi rezultatoj, ni povas akiri nenian evidentecon por tio ke -S-CO- specifa hidrolazo ekzistas, kaj sajnas ke tiuj ĉi S-esteroj estas hidrolizeblaj de hepato-O-esterazo mem kun ĝia malsevera substrato-specifeco.

6. Ni ne povis pruvi eksperimente fosforolizon de S-estero.

The authors are deeply indebted to Prof. T. Suzuki of Kyoto University for his generous gift of acetyl thiocholine and also to Dr. S. Isozima for his gift of atoxyl. The authors' thanks are also due to Dr. S. Yurugi for his guidance to the synthesis of methyl thioacetate.

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THE CHEMISTRY OF THE LIPIDS OF POSTHEMOLYTIC RESIDUE OR STROMA OF ERYTHROCYTES

V. GLYCOLIPIDS OF ERYTHROCYTES STROMA AND GANGLIOSIDE

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The authors have already reported that the glycolipids of red blood cells of mammals were very peculiar in nature. The glycolipid of equine blood stroma, named hematoside (*1*), is composed of lignoceric acid, sphingosine, two hexoses (mainly galactose) and N-acetylated prehemataminic acid; whereas human erythrocytes glycolipid, to which the name globoside was assigned, consists of lignoceric and nervonic acid, sphingosine, three hexoses (mainly galactose) and N-acetylchondrosamine (*2*, ADDENDUM).

Ganglioside, the well-known glycolipid in the grey matter of brain or spleen, had been found by Klenk (*3*, *4*) to comprise stearic or lignoceric acid, sphingosine, hexoses and neuraminic acid (=prehemataminic acid). The presence of chondrosamine in it was demonstrated through the work from Blix's laboratory (*5*) and was successively accepted by Klenk (*6*).

On the other hand, the present authors determined the distribution of lipid-hexosamine and lipid-hemataminic acid in the lyophilized blood stroma of various species of animals and thereby divided them into globoside type, hematoside type and mixed type (*7*). At that time, they suggested that ganglioside might be a mixture of globoside and hematoside in view of the presence of both lipid-hexosamine and lipid-hemataminic acid and because their contents are not stoichiometrical in Klenk's preparation: neuraminic acid (mol. wt., 281 or 311) 21 per cent, hexosamine hydrochloride (215) 10 per cent.

If ganglioside is a single substance and contains neuraminic acid and chondrosamine in a definite proportion, the ratio of their amounts would not be changed in the course of purification.

In order to elucidate the question as to whether or not ganglioside is a mixture, a pure sample was prepared from the grey matter of beef brain, and the contents of hexosamine and hemataminic acid were estimated in each step of purification. Electrophoresis was carried out for homogeneity judgement.

The final purest sample showed a single electrophoretic pattern and the contents of hemataminic acid (20 per cent) and hexosamine (10 per cent) were approximately equal to those reported by Klenk (*3*, *4* and *6*).

Both hematoside and globoside gave a single moving boundary at various pH in an electrophoretic apparatus; the mobility of the former was much faster probably due to its free carboxylic acid radical. In case of globoside, even in concentration of 0.5 per cent or lower, electrophoretic pattern could not be observed frequently through the irreversible jelly formation caused by the electric current. Such a phenomenon was described by Sharp *et al.* in the electrophoretic study of the egg-white inhibitor of influenza virus hemagglutination (*8*).

The aqueous solution of globoside and hematoside are extremely viscid; specific viscosity of 0.2 per cent aqueous solution of globoside, 0.504; hematoside, 0.274. Partial jelly formation like thixotropy often appeared in a 0.2 per cent solution of globoside when cooled overnight in an ice-box, and at the concentration more than 1 per cent the solution became almost gelatinous. Aqueous solution of ganglioside, however, flowed readily at this concentration.* The extremely high intrinsic viscosities of globoside (186 ml./g.) and hematoside (127 ml./g.) suggest the presence of extended, possibly thread-like, substances. In an analytical ultracentrifuge, each glycolipid sedimented with a single boundary; sedimentation constants of globoside, hematoside and ganglioside are 72 S, 70 S and 13.3 S, respectively.

The shape of sedimentation pattern of ganglioside was regularly symmetrical, while those of blood glycolipids were anomalous, suggesting probable formation of micellar structure in water. Furthermore, they gave extremely small diffusion constants.

Though it is still obscure whether these lipids are really high molecular polymer or only behave apparently as such in water, it is markedly interesting that ganglioside is distinctly different from the blood glycolipids in the physico-chemical properties, while ordinary nature is so similar that usual chemical treatment can hardly distinguish them.

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* Klenk was so sharp-sighted that he already noticed the concentrated aqueous solution of spleen ganglioside was "zähflüssiger" than brain ganglioside (*4*).

EXPERIMENTAL

Isolation of Ganglioside—The method employed for the isolation of ganglioside was essentially the same as that of Klenk *et al.* (*3, 4*). Twenty bovine brains obtained at the slaughterhouse were freed of membranes. Grey matter was obtained by gross dissection, triturated and dried from the frozen state. The dry material, 300 g., was extracted with a mixture of equal parts of dry methanol and ether for 5 days with occasional shaking. Four such extractions were carried out and the residue was extracted with chloroform-methanol in a Soxhlet apparatus. The light-yellowish extract was dissolved in hot methanol and methanolic cadmium chloride was added, filtered hot and bubbled with hydrogen sulfide. The hot-filtered solution was concentrated *in vacuo* and allowed to cool in an ice-box. The almost colorless powder which separated out (3.17 g. from ten brains, P 0.25) was dissolved in 20 ml. of pyridine and passed through a 15 cm.-long Brockmann's alumina column for the selective adsorption of sphingomyelin. From the filtrate and washings 2.42 g. of material was obtained. This was designated as B-1. B-1 was extracted with 95 per cent acetone for 10 hours in a small Soxhlet apparatus to remove cerebroside, residual portion being designated as B-2. This procedure was repeated several times, and hemataminic acid and hexosamine were estimated with each fraction as summarized in Table I.

TABLE I

Fractionation of Beef Brain Ganglioside

Fraction	Amount	Hemataminic acid	Hexosamine
	g.	*per cent*	*per cent*
B-1 (P 0.027)	2.42	4.33	2.66
B-2	1.6	6.56	3.61
B-3	0.95	8.98	4.73
B-4	0.35	12.29	5.64
B-5	0.27	15.67	7.27
B-6	0.25	17.72	8.54
B-7	0.20	18.18	8.90
B-8	0.12	18.79	9.47

B-8 was dissolved in hot methanol and allowed to cool overnight in an icebox. Colorless spherolithic crystals which separated out was designated as B-9. From the mother liquor, B-9-1 and B-9-2 were obtained.

Heated in a capillary tube, B-9-1 turned yellow at 190° and decomposed at 230°.

Analysis: The sample, B-9-1, was dried over P_2O_5 at 90°.

Diminution of weight: 8.44 per cent.

C 53.5, H 8.1, N 2.63, Hexosamine ·HCl 10.0, Hemataminic acid 20.2, Ash 1.5

Electrophoresis Studies—The electrophoretic studies were carried out in a modified

Tiselius apparatus (Hitachi, Model HT-B). Electrophoretic patterns of ganglioside have been obtained in phosphate buffer at pH 7.15 (ionic strength, 0.1). Potential gradient, 4.95 volt per cm. Electrophoretically more and more homogeneous preparations were obtained in the course of purification. Finally, single sharp ascending and descending boundaries were obtained. (Fig. 1).

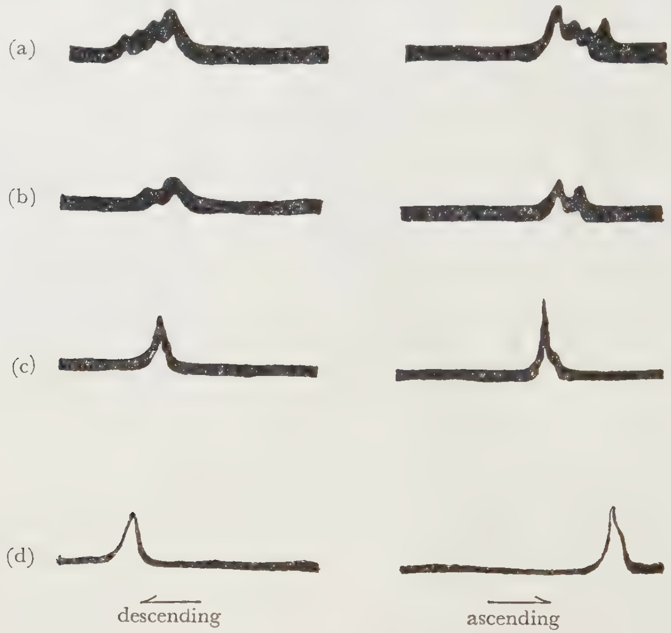

Fig. 1. Electrophoretic patterns of ganglioside preparations, (a) B-3, (b) B-5, (c) B-9-1, after 60 minutes; (d) Hematoside at pH 8.0 after 90 minutes.

Hematoside was electrophoretically homogenous at pH values from 3.8 to 9.95. Barbiturate, phosphate, acetate and glycine buffers were employed. Ionic strength was 0.1. Electrophoresis was carried out at a temperature of 2 to 3°. The conductivities of the buffer was used for mobility calculations. Fig. 2 shows the pH-mobility curve of hematoside.

Globoside gave a single but slower, anodic migrating boundary in the electrophoretic apparatus, but voluminous, gelatinous precipitates frequently developed and made the observation impossible. Sharp *et al.* had encountered the similar phenomenon in the study of egg-white inhibitior of influenza virus hemagglutination and explained it under electron microscope as the formation of fibrils from filaments caused by an orienting effect of the electric current (*8*).

Partial Specific Volume—This was determined pycnometrically at 20° on about 1 per cent concentration, the substances being dissolved in distilled water.

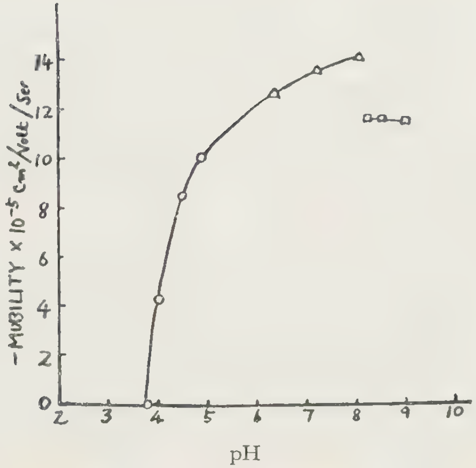

FIG. 2. pH-Mobility curve of hematoside
-○-○- Acetate, -△-△- Phosphate, -□-□- Veronal, × ×Glycine buffer.

Globoside 0.85, Hematoside 0.89, Ganglioside 0.87
The values obtained were higher than those previously found for many proteins. Nearer values were found on the substances of lipoidal nature; for instance, thromboplastic lipoprotein of lung, human serum α_1-globulin *etc.* (*9, 10*).

Viscosity Studies—Intrinsic viscosity measurements were carried out on the lipid dissolved in distilled water at 24.5±0.02° by means of an Ostwald viscometer. The results are recorded in Table II and Fig. 3. Specific viscosity of 0.05 per cent solution of globoside did not change as before, after heating at 95° for 50 minutes or preservation for one month in an ice-box. The effect of salt-addition on viscosity was very small.

TABLE II

Viscosity Studies of Glycolipids

Concentration (*g. per dl.*)	Globoside		Hematoside		Ganglioside
	Specific viscosity, η	η/c	Specific viscosity, η	η/c	Specific viscosity
0.2	0.504	2.53	0.274	1.37	0.0
0.1	0.212	2.12	0.132	1.32	
0.05	0.099	1.98	0.064	1.29	
0.025	0.054	2.24	0.032	1.28	
0.0125	0.029	2.32	0.02	1.60	
0	Intrinsic viscosity, H, $\lim_{c \to 0} \eta/c$	1.86 dl./g.		1.27 dl./g.	

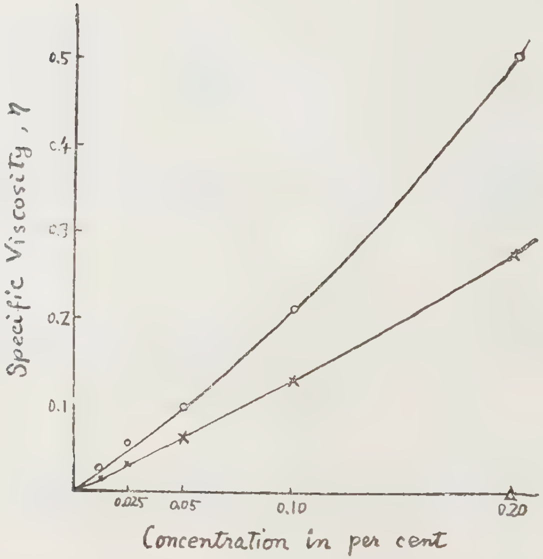

FIG. 3. Dependence of viscosity on concentration (○) Globoside (×) Hematoside (△) Ganglioside

The value of the viscosity increment, ν, calculated from the intrinsic viscosity, H, and partial specific volume, V, by the equation $\nu = 100\ H/V$, was found to be: Globoside 218, Hematoside 142. This corresponds to an axial ratio of about 55 and 45, respectively, provided they are elongated ellipsoid of revolution.

Sedimentation Studies—The ultracentrifugal experiments were performed in a Model E analytical ultracentrifuge of the Specialized Instruments Corporation. Temperatures from 16 to 19° were used during the runs. Sedimentation patterns of aqueous solution of three lipids are shown in Fig. 4. Addition of NaCl on the solution of globoside made it somewhat turbid and the pattern obscure (aggregation ?).

Table III summarizes the sedimentation constants of the lipids at different concentrations. Extrapolated values for zero concentration gave:

Globoside 72 S, Hematoside 70 S, Ganglioside 13.3 S.

Diffusion Studies—The diffusion coefficient of these glycolipids at 20° was kindly determined by Dr. N. Ui of the Institute of Science and Technology, the University of Tokyo. The diffusion pattern observed gave some indication of inhomogeneity. The results are shown in Table IV.

These data, when combined with each other, give a molecular weight of several ten millions for globoside and hematoside, whereas 8.7×10^5 (M_{sD}) for ganglioside. These are only the results of computation and the figures are unreliable. But it is a certain fact that these

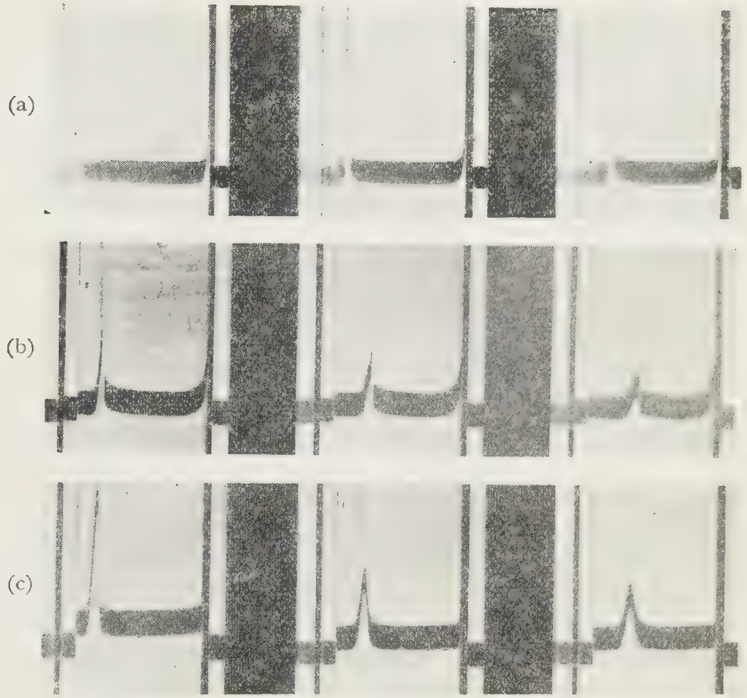

FIG. 4. Sedimentation pattern of glycolipids (0.8 per cent in water)
(a) Globoside, time intervals 8 minutes. Speed 26,100 r.p.m. Bar angle 70°
(b) Hematoside, time intervals 8 minutes. Speed 30,200 r.p.m. Bar angle 70°.
(c) Ganglioside, time intervals 8 minutes. Speed 50,900 r.p.m. Bar angle 60, 70° and 70°.

TABLE III

Variation of the Sedimentation Constant in Svedberg Units as a Function of the Lipid Concentration

Concentration	Globoside	Hematoside	Ganglioside
per cent			
0.8	38.5	53.8	11.7
0.4	54.9	62.3	12.4
0.2	63.9	—	12.9
0	(72)	(70)	(13.3)

TABLE IV

Diffusion Cofficients of Lipids

Sample	Solvent	$D_{20,w}\times 10^7$	$D_0\times 10^7$
		$cm^2 sec^{-1}$	
Globoside	Water	0.2_7 (gel-like)	
	Acetate-NaCl pH 5.0, $\mu=0.2$	0.1_3	0.08
Hematoside	″	0.6_0	0.5_2
Ganglioside	″	2.8	——

substances behave like high molecular substances.

DISCUSSION

Recently Klenk and Wolter (*11*) reported that they isolated from equine blood stroma a glycolipid with the same properties as hematoside reported by us (*1*). At the same time, Klenk and Lauenstein (*12*) obtained from human erythrocytes stroma a glycolipid which they already found in aged, powdered blood (*13*), and it is assumed by them as lignoceryl sphingosine acetylchondrosamine tri- or tetrahexoside. Just the similar composition had been also proposed by us to globoside as described previously (*2*). They demonstrated bovine erythrocytes contains both glucosamine-containing and neuraminic acid-containing glycolipids; the results are well agreed with ours that bovine blood stroma contains both lipid-hemataminic acid and lipid-hexosamine (*7*).

Klenk accepted that his neuraminic acid is the same substance as our prehemataminic acid, as they obtained neuraminic acid in equine blood glycolipid (hematoside) (*11*). Consequently, hemataminic acid is the same as methoxyl derivative of neuraminic acid, for which Klenk proposed $C_{11}H_{21}NO_9$, while $C_{10}H_{19}NO_8$ has been assumed by us. The terms, hemataminic acid and prehemataminic acid should be employed till this composition is conclusively decided. At that time, Klenk used the term ganglioside for hematoside probably because it comprised neuraminic acid. But we think the usage of the term, ganglioside, should be confined to the brain lipid, for the two lipids are categorically different in view of the present report.

According to the results of this paper, these glycolipids might be extremely high molecular substances, at least in the behavior in water.

The concept and treatment of high molecular lipid has already been shown in the study of Folch *et al.* on strandin (*14*). It is a glycolipid of brain grey matter and electrophoretically homogeneous, but the value of mobility reported by them is so high as to be reliable. The main sedimentation constant is nearly equal to that of ganglioside, but the partial specific volume of strandin, 0.76, seems too small seeing its lipoidal nature.

Globoside contains chondrosamine and no hemataminic acid, but the mucopolysaccharide fraction obtained from the delipidated stroma residue contains both and inhibits the influenza virus hemagglutination (Yamakawa and Ashihara, unpublished),—the fact which would support Klenk's assumption that neuraminic acid might play a role as virus receptor site (*15*).

Lately, globoside was found to be an agglutinogen of ABO system but devoid of VHI activity (*16*). Elinin, the lipoprotein of human erythrocytes, which is regarded as the carrier of Rh and ABO antigen, possesses the VHI activity, too (*17*, *18*).

It is considered globoside might be a lipidic constituent of elinin.

SUMMARY

1. In order to elucidate the question as to ganglioside is a mixture or a single substance, a pure specimen of ganglioside was prepared from lyophilized grey matter of bovine brain.

2. Ganglioside prepared by us was the same substance as that of Klenk and electrophoretically homogenous. Its solution in water flowed readily, while the aqueous solution of blood cell glycolipids were extremely viscous.

3. From the physico-chemical properties of aqueous solution, globoside, hematoside and ganglioside are likely to be highly polymerized substances.

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ON THE STRUCTURE OF LYSOZYME

I. QUANTITATIVE ESTIMATION OF CARBOXYL-TERMINAL AMINO ACID BY IMPROVED HYDRAZINOLYSIS METHOD

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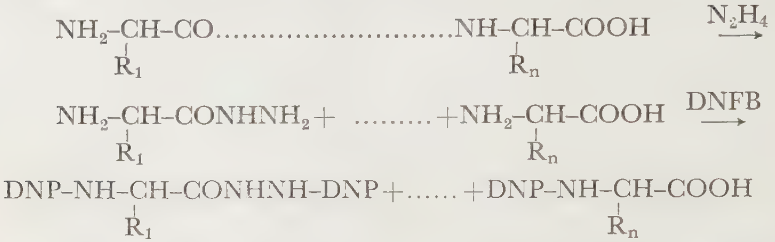
(Received for publication, August 27, 1953)

Lysozyme is an egg white protein having a lytic action to some bacteria. It has been extensively investigated chamically as well as physically in recent years. From the standpoint of its chemical structure, it has been already clarified that it consists of one peptide chain having about 125 amino acid residues (*1*, *2*), and that the amino acid sequences from its amino-terminal is lysylvalylphenylalanylglycyl...... (*3*). Many small peptides have also been detected from its inner molecule (*4–6*). However, its carboxyl-terminal amino acid has been investigated less satisfactorily, namely it has been characterized as leucine merely semi-quantitatively (*7–9*).

On the other hand, several methods for the characterization of carboxyl-terminal amino acids in proteins have been published in recent years (*10–13*), but none of them may satisfactorily be applied in common cases. We have reported a new method (*14*), in which proteins are hydrazinolysed into amino acid hydrazides and amino acids, the latter being derived from only carboxyl ends. In this way some ten proteins have been characterized qualitatively concerning their carboxyl ends (*9*). In the previous reports amino acids were separated from amino acid hydrazides by precipitating the latter with benzaldehyde as dibenzal compounds. However, this procedure was rather troublesome, and probably amino acids themselves were partly lost by being adsorbed in the precipitate, and some less soluble amino acids might be lost in the treatment. Another separation procedure was therefore necessary to make quantitative estimations of carboxyl ends.

As well known, F. Sanger (*15*) has published the dinitrophenyl (DNP) method for the characterization and quantitative estimation of amino terminal-amino acids in proteins. We applied this technique to separate amino acids from amino acid hydrazides, the latter of which gave di-DNP-derivatives substituted at their amino and hydrazide groups. The reactions involved are shown in the following scheme:



$$\mathrm{NH_2\text{-}\underset{R_1}{\underset{|}{CH}}\text{-}CO\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots NH\text{-}\underset{R_n}{\underset{|}{CH}}\text{-}COOH} \xrightarrow{N_2H_4}$$

$$\mathrm{NH_2\text{-}\underset{R_1}{\underset{|}{CH}}\text{-}CONHNH_2+\ \ldots\ldots\ldots+NH_2\text{-}\underset{R_n}{\underset{|}{CH}}\text{-}COOH} \xrightarrow{DNFB}$$

$$\mathrm{DNP\text{-}NH\text{-}\underset{R_1}{\underset{|}{CH}}\text{-}CONHNH\text{-}DNP+\ldots\ldots+DNP\text{-}NH\text{-}\underset{R_n}{\underset{|}{CH}}\text{-}COOH}$$

C-Terminal DNP-amino acids having free carboxyl groups can be quantitatively separated from the di-DNP-amino acid hydrazides by fractional extraction. The former are then separated and characterized chromatographically, and estimated colorimetrically.

In this present report lysozyme is treated as mentioned above and its carboxyl-terminal leucine is estimated to be one residue par mole lysozyme.

EXPERIMENTAL

Lysozyme—Lysozyme was prepared by the method of Alderton *et al* (*16*). It was recrystallized five times through carbonate, hydrochloride and isoelectric crystals and the final isoelectric crystals were dialyzed against distilled water and evaporated *in vacuo*.

Anhydrous Hydrazine—Anhydrous hydrazine was prepared as follows: Dilute hydrazine hydrate was prepared from hydrazine sulfate by treating with potassium hydroxide (*17*). It was concentrated to above 85 per cent by distilling off water with xylene (*18*). The concentrated hydrazine hydrate was then made anhydrous by treating with potassium hydroxide and by distillation (*19*). To avoid the contamination with potassium hydroxide, the anhydrous hydrazine was redistilled under reduced pressure. Thus anhydrous hydrazine, its concentration being above 95 per cent, was obtained. All the concentrations were checked by titrating with potassium bromate solution (*20*).

Hydrazinolysis and Dinitrophenylation—Ten mg. of lysozyme were hydrazinolysed with about 0.3 g. of anhydrous hydrazine at 100°. Lysozyme was dissolved soon in the medium. The time of hydrazinolysis was varied from 5 to 20 hours. After the reaction mixture was evaporated *in vacuo* over sulfuric acid to remove excess hydrazine, the residue was dissolved in 10 ml. of 5 per cent sodium bicarbonate solution, followed by adding 20 ml. of ethanolic solution containing 0.5 ml. of dinitrofluorobenzene. Shaking the reaction mixture for 3 hours in a dark room at room temperature, it was concentrated under reduced pressure, and the residue was taken up with about 30 ml. of water. After adding a sufficient amount of hydrochloric acid to acidify it, the yellow suspension was transferred to a separating funnel from the reaction vessel, the latter being washed by several portions of ethyl acetate. The combined ethyl acetate was transferred to the separation funnel and shaken with the reaction mixture. On further two or three extractions with ethyl acetate, the suspension was all dissolved in them and the water phase became almost colorless. The yellow ethyl acetate extracts were combined and shaken with five equal volumes of 4 per cent sodium bicarbonate solution, when DNP-amino acids derived from C-terminal and, if any, α,α-di-DNP-Asp- and/or Glu-hydrazide derived from α-aspartyl and/or α-glutamyl residues in peptide were extracted to the bicarbonate solution, and other di-DNP-hydrazides remained in the ethyl acetate with brown red color. After the bicarbonate extract was washed with small amount of ethyl acetate, it was acidified with hydrochloric acid and re-extracted with three small portions of ethyl acetate. The extract was combined and filled up to a definite volume, divided into equal three portions, and each was taken to dryness *in vacuo*. Chromatographic separations and colorimetric estimations were carried out with these three portions separately.

Chromatographic Separation and Quantitative Estimation of C-terminal Amino Acid—The separation of DNP-amino acids was carried out by silica gel chromatography described by Sanger (*15*), although some modifications were made with developing solvent according to the nature of silica gel prepared by us. We used butanol-chloroform system as mobile phase and 2 or 10 per cent acetic acid as stationary phase.

The final extract of the hydrazinolysate was separated on the column into several bands. Most strong band was identified as DNP-leucine and others contained DNP-compounds of C-terminal peptides and aspartic acid hydrazide. Further, unconfirmed bands, probably decomposition products of DNP-amino acid, were also detected. All these bands, except that of DNP-aspartic acid hydrazide, were so little that could not be confirmed definitely. The band of DNP-leucine was cut off, eluted with ethyl acetate, taken to dryness *in vacuo*, dissolved in 5 to 10 ml. of 1 per cent sodium bicarbonate solution, and estimated colorimetrically using photoelectric colorimeter at wave length of 420 mμ.

All the treatments after the dinitrophenylation were carried out under shutting off from direct rays of the sun in order to avoid photolysis of DNP-derivatives (*21*).

The amounts of C-terminal leucine, liberated from lysozyme in various times of hydrazinolysis are listed in Table I, which shows that the amount of leucine increases to a maximum value at about 12 hours and then decreases slowly on further hydrazinolysis. This observation indicates that the leucine is decomposed partly in the course of hydrazinolysis. Therefore, some control experiments must be carried out in order to estimate the true amount of leucine located at the C-terminal.

Control Experiments—To find a correction factor for the decomposition of leucine, leucine was " hydrazinolysed " for various periods of time under the same conditions, the " hydrazinolysate " was treated in the same manner described above, and recovery of leucine was estimated quantitatively. The decomposition factor computed from the recovery is shown in Table II. It seems very curious that the decomposition during hydrazinolysis is so remarkable, but this is comparable with the decomposition of DNP-amino acids during hydrolysis in the case of determining amino-terminal. Although the manner of decomposition of leucine is not yet clear, some unconfirmed faint bands,

TABLE I

*Estimation of the Carboxyl-terminal Leucine of Lysozyme**

Time of hydrazinolysis (*hrs.*)	Lysozyme		C-Terminal leucine liberated		
	(*mg.*)	($10^{-7}M$)	Found ($10^{-7}M$)	Leucine / Lysozyme (uncorrected)	Leucine** / Lysozyme (corrected)
5	10.00	6.66	1.74	0.26	(0.48)***
7.5	10.05	6.70	3.31	0.49	(0.73)
10	20.10	13.40	7.40	0.55	0.90
10	104	71	42.6	0.60	0.95
10	101.8	67.9	46.2	0.68	1.03
12	100.0	66.6	43.6	0.68	1.06
20	9.95	6.63	3.51	0.53	
20	10.55	7.04	3.38	0.48	

* The molecular weight of lysozyme is assumed to be 15,000.

** Corrected by the factor cited in Table II.

*** Values in parentheses are corrected by the factors interpolated by the curve of Fig. 1.

TABLE II

Recovery of Leucine after "Hydrazinolysis."
(*Leucine only*)

Time of hydrazinolysis (*hrs.*)	Initial leucine		Recovered leucine		Decomposition factor
	(*mg.*)	(μM)	(μM)	Recovery factor	
1	0.561	4.28	4.10	0.96	0.04
9	0.472	3.60	2.35	0.66	0.34
10	1.032	7.89	5.19	0.66	0.34
10	1.080	8.24	5.31	0.65	0.35
15	0.533	4.06	2.40	0.59	0.41

which are detected in the hydrazinolysate of lysozyme, are detected on silica gel chromatogram.

Being corrected from the data cited in Table II, the true amounts of leucine liberated from lysozyme in the course of hydrazinolysis are shown in the last column of Table I. From these figures it is clear that lysozyme has one leucine residue at its C-terminal, and that the leucine is liberated fully in 12 hours' hydrazinolysis.

Further following control experiments were carried out: Lysozyme and nearly equimolecular amount of leucine were together hydrazinolysed under the same conditions as above, and the recovery of leucine was estimated. Results obtained, given in Table III, are in good agreement with those obtained from the former experiment

TABLE III

Recovery of Leucine after "Hydrazinolysis."
(Leucine plus lysozyme)

Time of hydrazinolysis (*hrs.*)	Lysozyme ($10^{-7}M$)	Leucine ($10^{-7}M$)	Total ($10^{-7}M$)	Estimated leucine	
				Total ($10^{-7}M$)	Recovery factor
10	60.9	65.6	126.5	80.6	0.60
10	13.3	15.6	28.9	16.1	0.56
15	6.84	7.63	14.47	8.32	0.57

assuming that lysozyme has one C-terminal leucine. 1 shows the true leucine liberated from lysozyme during various times of hydrazinolysis.

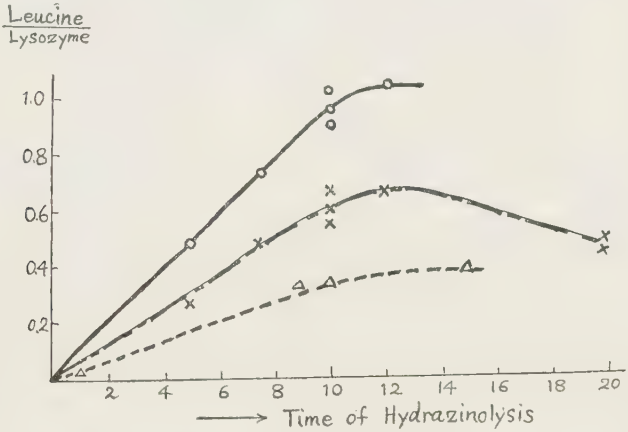


FIG. 1 Hydrazinolysis of lysozyme.
○——○ Corrected amount of leucine
×——× Found leucine
△- - - -△ Decomposition factor of leucine

DISCUSSION

Comparing this method to the DNP-method of N-terminal, the former differs from the latter in the following points:

1. Proteins are hydrazinolysed and then dinitrophenylated in the case of C-terminal, while they are hydrolysed after dinitrophenylation in the case of N-terminal.

2. Whereas lysine residues in peptide chains appear as ε-DNP-derivative in the latter case, glutamic and aspartic acids in peptides are extracted in acidic fraction as α,α-di-DNP-glutamic and aspartic acid-α-hydrazides in the former case. If glutamine or asparagine occupies C-terminal, or if glutamic or aspartic acids exists in peptide as γ- or β-peptides, they are expected to form α,γ-di-DNP-glutamic acid-γ-hydrazide or α,β-di-DNP-aspartic acid-β-hydrazide, respectively, whereas those which exist in peptide chains as amides may become tri-DNP-glutamic or aspartic acid-di-hydrazides, which are not extracted in acidic fraction. In this way glutamic and aspartic acids located in peptide chains whose one carboxyl group is free might be estimated. On this account investigations are now in progress.

3. As in the case of N-terminal, the hydrazinolysis-DNP-method may be applied to characterize the C-terminal peptide by partial hydrazinolysis. Preliminary experiments on this problem promised its availability. Detail results will be published later.

SUMMARY

1. An improved method for characterization and estimation of C-terminal amino acids in proteins, by means of hydrazinolysis and dinitrophenylation, is described.

2. Lysozyme is proved to possess one leucine residue at its C-terminal.

The author wishes to express his gratitude to Professor S. Akabori for his kind guidance and encouragements through this investigation, to Mr. K. Narita who supplied DNFB and DNP-amino acids, and also to Mr. A. Nagata for his much technical assistance.

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BACTERIAL SYNTHESIS OF AROMATIC METABOLITES

I. SYNTHESIS OF A LABILE PRECURSOR OF PHENYLALANINE BY NON-PROLIFERATING CELLS OF AN AUXOTROPH OF ESCHERICHIA COLI

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(Received for publication, September 7, 1953)

In 1950, Simmonds (*1*) reported an accumulation in culture media by a phenylalanineless mutant of *Escherichia coli* of an unidentified growth factor (for itself). Recently it has been confirmed by Davis (*2*) and by Katagiri and Sato (*3*) that such a growth factor accumulated in culture filtrates of certain phenylalanine auxotrophs is phenylalanine itself. The mechanism underlying this unusual accumulation has been elucidated by Katagiri and Sato (*3*), who showed that shaking of the mutant cells with Davis' minimal medium (*4*) resulted in an accumulation of an unstable precursor of phenylalanine, provisionally called Compound X. Although this precursor was not active for the growth of the mutant, it could be readily converted to Compound Y, an active precursor, by heat. Due to its instability, however, it is converted during prolonged cultivation. The latter once formed, can rapidly give rise to the phenylalanine under the influence of the enzyme system present in the mutant cells. Essentially the same observation has independently been attained by Davis (*2*, *5*) showing that young cultures of the auxotrophs do contain an acid-labile precursor of phenylalanine (called prephenylalanine). He has further shown that relatively stable accumulation of this substance could also be obtained in media heavily buffered at slightly alkaline reactions. He also proved that the active compound, derived from prephenylalanine, was phenylpyruvic acid.

It seemed to be noticeable that non-proliferating cells of the auxotroph can easily synthesize Compound X (probably identical with Davis' prephenylalanine) during a short period of shaking, since this may provide experimental convenience to investigate the enzyme system concerned in aromatic biosynthesis. Recent work using this shaking technique has shown that the synthesis of Compound X by the cells requires only four substances, namely glucose, $K^{\cdot}$, $NH_4^{\cdot}$, and inorganic phosphate. The present report describes several characteristics of the synthetic process.

MATERIALS AND METHODS

Organisms—Phenylalanine-requiring mutants (*e.g.* M-Ph601) tryptophan-requiring mutants (*e.g.* M-Tr459) and shikimic acid-requiring mutant (M-Sh508) were employed. All were derived in our laboratory by the penicillin method of Davis (*6*) from *E. coli* in the culture collection of this laboratory.

Shaking Procedure—The mutants grown on a minimal medium (*4*) (consisting of glucose, citrate, potassium phosphate, ammonium sulfate and magnecium sulfate) solidified with agar and supplemented with 50 μg. per ml. of phenylalanine. After 18 hours cultivation, the cells were harvested and washed thrice with physiological saline. With the exception noted, the washed cells (5×10^9 cells per ml.) were suspended in $M/15$ potassium phosphate buffer (pH 7) containing $M/50$ glucose and $M/100$ ammonium chloride, and shaken at 37° for one hour.

Assay for Compound X—After the shaking procedure, the suspensions were centrifuged, the supernatants were filtered with a Seitz filter and the filtrates were autoclaved at 120° for 10 minutes in order to convert the Compound X to the active factor (Compound Y). The amount of Compound Y in the autoclaved filtrates were determined by bioassay with M-Ph601 as assay organism. M-Ph601 was inoculated in the sample diluted with the equal volumes of the double strength minimal medium and grown for 18 hours at 37°. The growth of the mutant were then measured tubidimetrically with a Hitachi photoelectric colorimeter (Model A). Since Compound X is completely converted to Compound Y by the autoclaving procedure, the growth responses of the mutant on the autoclaved filtrates were assumed to be proportional to the amounts of Compound X synthesized during shaking. In the following, therefore, the amounts of Compound X will be expressed in terms of optical density of the assay cultures.

RESULTS

Phenylalanine Accumulation—To make sure that the phenylalanine auxotroph accumulates the growth factor for itself, the following dilution method was applied. M-Ph601 was grown at 37° for 24 hours in the minimal medium supplemented with a limited amount (3 μg. per ml.) of phenylalanine. The Seitz filtrate of this culture (F601-1) was reinforced with 1.5 volumes of the fresh phenylalanine-free double strength minimal medium and then autoclaved. M-Ph601 was again grown to this reinforced filtrate. The culture filtrate thus obtained was designated as F601-2. This filtrate was again diluted with the double strength minimal medium to the same extent as before and M-Ph601 was again grown; the filtrate of this culture was F601-3. Thus, repeating this procedure, F601-8 was finally obtained. This final filtrate was found to be as effective as F601-1 in supporting the growth of M-Ph601, though it must contain, as a result of serial dilution, only a negligible amount of the originally supplied phenylalanine (at most $3\times(1-0.4)^{8-1}$ μg. per ml.). Typical results of these experiments are shown in Table I.

TABLE I

Response of M-Ph601 to the Culture Filtrate of M-Ph601

Culture filtrate*	F601–1	F601–2	F601–3	F601–4	F601–5	F601–6	F601–7	F601–8
Growth of M-Ph601	0.45	0.40	0.43	0.37	0.32	0.40	0.46	0.42

* Refer to the text.

The culture filtrates were reinforced with 1.5 volumes of the fresh phenylaninefree double strength minimal medium and M-Ph601 was then grown on them. After 18 hours, the growth of the mutant was measured and expressed in terms of optical density units (at 570 mμ).

Although the facts reported above confirmed the accumulation of a substance in the filtrates which is responsible for the growth of the mutant, it was still uncertain whether it is phenylalanine or its precursor. However, decisive evidence that the substance involved is actually phenylalanine was obtained by paper chromatographic analysis of F601–8. The paper chromatogram with butanol-acetic acid-water and lutidine-collidine showed the presence of phenylalanine together with four other unidentified amino acids by ninhydrin test. Furthermore, it was confirmed that M-Ph601 does respond only to the eluate from the paper strip corresponding to the phenylalanine spot.

Synthesis of Labile Phenylalanine Precursor—Washed cells of M-Ph601 harvested from the solid minimal medium supplemented with phenylalanine (50 μg. per ml.) were suspended in the minimal medium and shaken at 37°. After one hour of shaking, the suspensions were filtered with a Seitz filter. The filtrate (reinforced with double strength minimal medium) failed to support the growth of the mutant. It is obvious that this emergence of the growth-supporting activity is not due to the destruction of an inhibitor by heat, since M-Ph601 was found to be able to grow vigorously in the non-autoclaved Seitz filtrate if equal volumes of autoclaved filtrate were added (Table II).

TABLE II

Response of M-Ph601 to the Medium Obtained after Shaking the Cell of M-Ph601 in the Minimal Medium

Medium	Seitz filtrate (autoclaved)	Seitz filtrate (not autoclaved)	Seitz filtrate (autoclaved+not autoclaved) 1:1
Growth of M-Ph601	0.25	0.02	0.13

The Seitz filtrates were reinforced with equal volumes of the fresh double strength minimal medium. The growth was measured after 18 hours cultivation and expressed in the optical density units (at 570 mμ).

Among the constituents of the minimal medium only four *viz.* glucose, potassium ion, ammonium ion and inorganic phosphate were indispensable for the synthesis of Compound X under the experimental conditions employed. The concentrations of the factors required for maximal synthesis of Compound X were determined from the data recorded in Fig. 1. In the following experiments, therefore, a solution containing $M/50$ glucose, $M/100$ ammonium chloride and $M/15$ potassium phosphate was used as shaking medium. Nitrate ion could replace ammonium ion to some extent as shown in

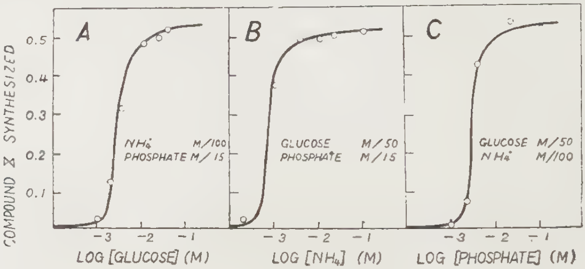

Fig. 1. Effect of concentrations of glucose (A), NH_4^+ (B), and potassium phosphate (C) on the synthesis of Compound X. Shaking conditions: 37°, pH 7.0, 60 min. Shaking filtrates were diluted with equal volumes of the double strength minimal medium and autoclaved. M-Ph601 was then grown on them for 18 hrs. The amount of Compound X was expressed in terms of the growth (optical density at 570 mμ) of the mutant.

Table III

Effect of Various Substarates on the Synthesis of Compound X

Substrate*	Compound X synthesized
Glucose+NH_4Cl	0.30
Glucose+$NaNO_3$	0.25
Sodium shikimate+NH_4Cl	0.02
Sodium shikimate+ATP+NH_4Cl	0.02
Sodium shikimate+sodium lactate+NH_4Cl	0.02

Shaking conditions: at 37°, in $M/15$ potassium phosphate buffer at pH 7.0, for 60 minutes. The shaking filtrates were diluted with an equal volume of the double strength minimal medium and autoclaved. M-Ph601 was then grown for 18 hours in them. The amount of Compound X was expressed in terms of the growth (optical density) of the mutant.

* Concentration of 0.01 M.

Table III. No significant synthesis of Compound X can be achieved in the shaking media in which glucose is replaced by shikimic acid[1] (0.01 *M*) alone or this acid plus such an energy source, as adenosine triphosphate (0.01 *M*) or lactate (0.01 *M*). These are shown in Table III, too.

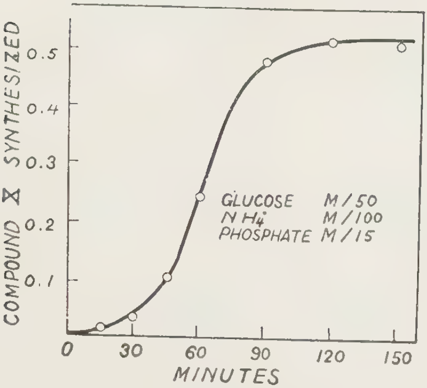

FIG. 2. The synthesis of Compound X as a function of time. Shaking conditions: 37°, pH 7.0. The amount of Compound X was measured and was expressed as in Fig. 1.

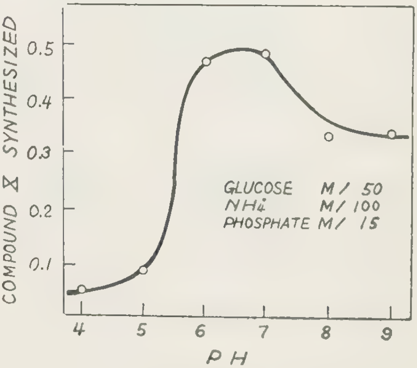

FIG. 3. Effect of pH upon the synthesis of Compound X. The amount of Compound X was measured and was expressed as in Fig. 1.

As will be seen in Fig. 2, the formation of the precursor at pH 7 reached a maximum after about 2 hours shaking. An induction period of 20–30 minutes was invariably observed.

The synthesis occurred most rapidly at pH 6–7 (Fig. 3).

Penicillin (500 units per ml.) exhibited little effect on the process; carbon monoxide and cyanide were inhibitory. An appreciable synthesis of Compound X was seen under anaerobic conditions (Table IV).

Further, it has been noted that the cells grown on peptone-broth-agar media are less effective or often inactive under these shaking conditions; remarkable synthesis can be attained only with the cells grown on the minimal medium-agar supplemented with phenylalanine, the reason of which is not clear.

Compound X and Compound Y—As shown above, shaking of the cells of M-Ph601 with potassium phosphate buffer containing glucose and $NH_4^{\cdot}$ results in an accumulation of Compound X, which has no growth factor activity, as previously shown by Davis (*5*), either for phenylalanine auxotrophs or, in the presence of multiple aromatic supplement

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1) Isolated by Mr. Mitsuo Ebata of Kanazawa University from dried fruits of *Illicium religiosum*. The author is indebted to him for furnishing this material.

TABLE IV

Effectors on the Synthesis of Compound X

Effector	Compound X synthesized
Penicillin (500 units per ml.)	0.27
CO (saturated)	0.01
KCN ($M/1000$)	0.01
Anaerobic conditions	0.20
None	0.30

Shaking conditions: 37°, pH 7.0, 60 min.

Shaking media: $M/50$ glucose and $M/100$ NH_4Cl in $M/15$ potassium phosphate buffer. The amount of Compound X was measured after removing these inhibitors and expressed as in Table I.

other than phenylalanine, for a mutant with earlier blocks (M-Sh508). This mutant requires shikimic acid or multiple aromatic supplement (Table V).

TABLE V

Response of the Mutant (M-Sh508) to Compound X

Supplement*	Basal medium	Growth
Tyrosine+tryptophan+*p*-aminobenzoic acid +phenylalanine	Minimal medium	0.35
Tyrosine+tryptophan+*p*-aminobenzoic acid	Shaking filtrate**	0.17
None	Shaking filtrate**	0.16
Sodium shikimate	Minimal medium	0.50

* Supplemented to the concentration of 30 μg./ml. in minimal medium.

** Reinforced with equal volumes of double strength minimal medium.

The growth of M-Sh508 was measured and expressed as in Table II.

The accumulated Compound X is so unstable that it is readily converted to the active principle (Compound Y) by heat (Fig. 4) or by acidifying the filtrate (Fig. 5). The Compound Y thus formed can be extracted from the acidified filtrate by ether. Davis (5) has partly purified his prephenylalanine and has identified its active product as phenylphruvic acid. The Compound Y obtained by the author seems to be also identical with phenylpyruvic acid.

Another Growth Factor and Another Mutants—It was further noted that the medium obtained after shaking the suspension of the cells of M-Ph601 contains, other than Compound X, a factor which feeds another mutant (M-Sh508). Such a substance was also found in the medium of a tryptophan auxotroph (M-Tr459).

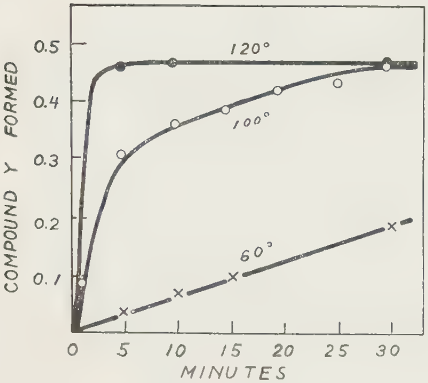


Fig. 4. Activation of the shaking filtrate by heat. Each sample was diluted with an equal volume of the double strength minimal medium and the amount of formed Compound Y (phenylpyruvic acid) was expressed in terms of the growth (optical density) of the mutant.

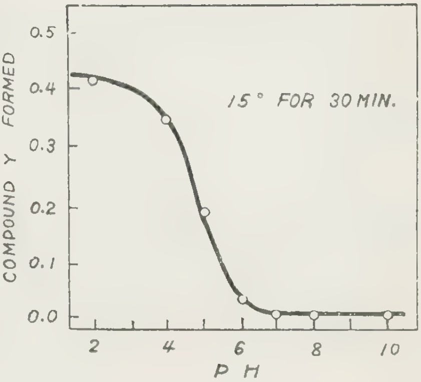


Fig. 5. Activation of Compound X by acid. Formed Compound Y (phenylpyruvic acid) was measured and was expressed as in Fig. 4.

It is noticeable that the parent wild type strain, tryptophanless mutants and shikimic acid requiring mutants all failed to accumulate Compound X in the filtrates under the above conditions (Table VI).

TABLE VI

Response of Various Mutants to the Shaking Filtrates of Various Mutants

Response of / Filtrate of	M-Ph601	M-Sh508	M-Tr459	Wild type
M-Ph601	+	+	—	+
M-Sh508	—	—	—	+
M-Tr459	—	+	—	+
Wild type	—	—	—	+

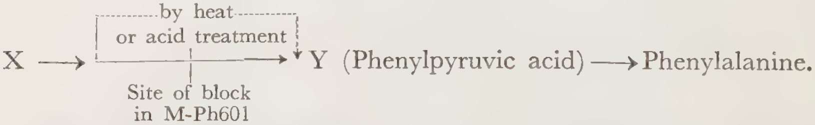
+ : Normal growth. — : No growth

Shaking conditions: 37°, pH 7.0, 60 min.

Shaking media: $M/50$ glucose and $M/100$ NH_4Cl in $M/15$ potassium phosphate buffer. The shaking filtrates were diluted with equal volumes of the double strength minimal medium and autoclaved. Each mutant was then grown for 18 hours in them.

DISCUSSION

Phenylalanine Accumulation—The above-mentioned conclusion that the phenylalanine auxotrophs accumulate the very phenylalanine in the culture filtrate may seem rather strange, since, according to the current theory, the mutant exacting for a substance has a genetic block at a certain reaction in the biosynthesis of that substance. A possible explanation for this phenomenon, however, could be found by the following observations. That is, shaking of the cells of the mutant with potassium phosphate buffer containing glucose and ammonium ion results in an accumulation of Compound X which is so unstable that it is readily convertible by heat or other factors to Compound Y, the product of the blocked reaction. Thus the filtrate originaly contains only Compound X, substrate of the blocked reaction and therefore is inactive. After heating, however, Compound Y is produced and thus the filtrate aquires the activity. These relations might be readily understandable by the following scheme:



From these shaking experiments it may be possible to explain the results of the culture experiments as follows. In the culture experiments, too, the accumulation of Compound X does take place as the first sequence (Davis (*2, 5*)). Due to its instability, however, it is gradually converted to Compound Y during the prolonged incubation.

Synthetic Process of Compound X—Among the four factors, glucose serves, of course, as the source of the carbon skeleton of the precursor, It has already been shown that glucose is directly utilized by yeast as a carbon source for the synthesis of phenylalanine and tyrosine in preference to acetic acid or other organic acids of the tricarboxylic acid cycle (*7, 8*). However, the possibility also exists that part of the utilized glucose may be oxidized or fermented to supply energy in the form of high-energy phosphate bond to the synthetic mechanism. The necessity of inorganic phosphate and potassium ion may be regarded as a manifestation of such a situation; or it is also probable that some phosphorlyated intermediates may be involved in the process. The role of ammonium ion is rather difficult to explain, since phenylpyruvic acid (and possibly also Compound X) is not a nitrogeneous compound. A relatively low concentration of this ion required suggests a catalytic function. However, the possibility that nitrogen might be attached to the carbon skeleton on some intermediary stages could not be excluded, since α-phenylglycine has been suggested as a precursor of phenylalanine in *Neurospora* (*9*). At any rate it may be expected that further pursuit of the functions of the three factors by our shaking technique would yield important informations on the mechanims of aromatic biosynthesis.

Davis (*10*) has established that shikimic acid is a common precursor of a number of aromatic metabolites including tyrosine, phenylalanine, tryptophan, *p*-aminobenzoic acid and *p*-hydroxibenzoic acid. From the above finding, it seemed of considerable interest to test the possibility that shikimic acid with or without energy sources (other than glucose) might replace glucose in our system. The experiments, however, showed that no significant synthesis of Compound X can be achieved in the shaking media in which glucose is replaced by shikimic acid alone or this acid plus an energy source, *e.g.* ATP or lactate. Furthermore, Compound X has no growth factor activity for a mutant with earlier blocks (M-Sh508) even in the presence of all required factors other than phenylalanine. The reasons for these negative results remained unsolved.

SUMMARY

1. There was ascertained by bioautography the accumulation of phenylalanine in the culture medium of a phenylalanineless mutant (M-Ph601) of *E. coli.*

2. It was further found that the synthesis of an unidentified and labile precursor (Compound X) of phenylalanine occurred in the suspension medium by shaking the resting (non-proliferating) cells of M-Ph601 with glucose, $K^{\cdot}$, $NH_4^{\cdot}$ (or NO_3') and inorganic phosphate, neither of which were dispensable for the synthesis.

3. The effects of the concentration of these supplements, and of pH on the synthesis were investigated. The synthesis did not occurred with shikimic acid and ATP in place of glucose. It was inhibited by CO and CN', but not by penicillin.

4. Compound X had no ability to support the growth of M-Ph601 and was readily convertible by heat or acid-treatment to Compound Y, which seemed to be phenylpyruvic acid and was capable of supporting the growth of M-Ph601.

5. The synthesis of Compound X was accomplished only with the

cells grown on a minimal medium containing phenylalanine and not with the cells grown on peptone-broth-agar media.

6. The medium obtained after shaking the suspension of M-Ph601 or tryptophan requiring mutants (*e.g.* M-Tr459) with above-menttioned supplements, also contained a factor capable of supporting the growth of a shikimic acid requiring mutant (M-Sh508) of *E. coli.*

RESUMO

1. Estis eltrovite pere de bioautografado ke la akumulado de fenilalanino en la kulturmedio de unu fenilalanino-benzonanta mutanto (M-Ph601) de *E. coli.*

2. Estis plie eltrovite ke la sintezo de unu neidentigata kaj malstabila antaŭulo (Kombinaĵo X) de fenilalanino okazis en la suspend-medio per la skuado restantaj ĉeloj de M-Ph601 kune kun glukozo, $K^{\cdot}$, $NH_4^{\cdot}$ (aŭ NO_3') kaj neorganika fosfato, kiuj estis nepre necesaj por la sintezo.

3. Efektoj de koncentrecoj de tiuj ĉi aldonatoj, kaj de pH sur la sintezo estis esploritaj. La sintezo ne okazis kun ŝikimika acido kaj adenosintrifosfato anstataŭ glukozo. CO kaj CN' malhelpis ĝin, dum penicilino montris neniom da influo.

4. Kombinaĵo X estis facile konvertebla al Kombinaĵo Y per varmigado aŭ per aldonado de acido, kiu estis kapabla subteni kreskadon de M-Ph601. Kombinaĵo Y sajnas esti fenilpiruvika acido.

5. Sintezo de Kombinaĵo X okazis nur kun ĉeloj kreskintaj sur minimum-medio kun fenilalanino, dume ĝi ne okazis kun ĉeloj kreskintaj sur peptono-buljono-agaragara medio.

6. La medio akirita skuinte suspendaĵon de M-Ph601 aŭ unu triptofan-benzonanta mutanto (M-Tr459) kun super-diritaj aldonatoj, enhavis ankaŭ factoron kapablan subteni kreskadon de unu ŝikimika acido-benzonanta mutanto (M-Sh508) de *E. coli.*

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AMINO ACID COMPOSITION OF MYCOBACTERIUM TUBERCULOSIS H37Rv[1]

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Until the fairly recent development of micro methods of analyses, investigators interested in the determinations of the amino acid contents of the tubercle bacillus were hampered by the difficulties involved in obtaining a large enough quantity of bacterial mass to work with. Stein and Moore (*1*) demonstrating the applicability of their chromatographic ninhydrin procedures to the problem, first published a complete (except for cystine) amino acid analysis curves of defatted cells of *Mycobacterium tuberculosis* H37Rv and H37Ra. Following this Eguchi (*2*) published his results of a complete analysis of the amino acids of a human strain of tubercle bacillus. He used a specific method for the analysis of each amino acid. Previously the contents of specific amino acids in the cellular proteins of various strains of tubercle bacilli were investigated by several other researchers beginning with Tamura in 1913 (*3–9*).

The present report is on the amino acid analyses of defatted cells of *M. tuberculosis* H37Rv, streptomycin sensitive and streptomycin resistant variants, by the chromatographic ninhydrin methods of Moore and Stein (*10–13*). Also, a few analyses of the tuberculin protein are included. Seibert (*14*) has recently published the analyses of the amino acid contents of purified proteins A and C of tuberculin.

EXFERIMENTAL

Mycobacterium tuberculosis H37Rv streptomycin sensitive and streptomycin resistant (H37RvSR) variants[2] were cultivated in Proskauer-Beck liquid synthetic medium for several weeks. The cells were filtered out, and defatted with 70 per cent ethanol, 95 per cent ethanol, 3:1 ethanol-ether, 1:1 ethanol-ether, ether, 1:1 ether-chloroform,

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[1] This work was supported in part by a grant from the Los Angeles County Tuberculosis and Health Association, Los Angeles, California.

[2] Both strains were kindly supplied by William Steenken, Jr. of the Trudeau Foundation, Trudeau, New York.

and chloroform in that order. Weighed portions of the dried defatted bacterial cells were hydrolyzed with constant boiling HCl solution (2.0 ml. per mg. of bacteria) by refluxing for 16 to 20 hours in boiling water bath or in an aluminum heating block. The hydrolysates were evaporated to dryness under a stream of air, redissolved in the chromatogram solvent, filtered, evaporated to dryness again, and stored in the deep freeze until chromatogramed.

The starch and Dowex 50 chromatographic ninhydrin procedures of Moore and Stein (*10–13*) were used with slight modifications. In the starch column analyses the the fractions were evaporated to dryness to remove the HCl instead of neutralizing with NaOH solution in preparation for ninhydrin testing, and in the Dowex chromatographic analyses, in order to cut down on the amount of work required, the amino acid peaks were located by filter paper spot ninhydrin tests of the fractions, and the fractions of each peak were pooled partially, and the quantitative ninhydrin analyses were done on a measured portion of each pooled sample.

Filter paper spot ninhydrin: With a micropipette 0.0025 ml.-portion of each fraction collected from the chromatographic column was spotted onto strips of filter paper which had been ruled off into small squares and labeled with the fraction numbers. The strips were air dried, dipped into water saturated butanol ninhydrin solution, air dried again, and hung in a hot air oven to develop the ninhydrin color.

RESULTS

The results are presented in the accompanying tables.

TABLE I

Relative Molar Concentration of Amino Acids

(Starch column analyses of tuberculin protein)

Amino acids	Chromatogram No. 1	2	3
Leucine + isoleucine	—	8.9	12.9
Phenylalanine	—	1.3	2.9
Valine + methionine	11.0	2.8	11.3
Tyrosine	1.2	2.3	1.2
Proline	2.4	2.5	2.5
Glutamic acid + alanine	22.0	22.0	21.9
Threonine	5.5	5.8	5.9
Aspartic acid	6.0	6.3	3.7
Serine	6.0	6.0	6.0
Glycine	9.3	9.6	8.9
Arginine + NH_3	15.0	15.7	16.5
Lysine	—	6.6	4.6
Histidine	—	—	0.3
Cystine	—	—	1.2

TABLE II

Relative Molar Concentration of Amino Acids

(Starch column analyses of *Mycobacterium tuberculosis* H37Rv cells)

Amino acids	Chromatogram No. 4	5	6	7	8	9
Leucine + isoleucine	9.6	15.8	15.1	12.4	12.8	11.7
Phenylalanine	1.9	3.2	3.3	3.3	2.1	3.4
Valine + methionine	10.7	10.7	11.1	10.4	7.4	13.6
Tyrosine	1.5	1.5	1.6	1.5	0.5	2.1
Proline	2.1	2.1	2.1	2.2	2.6	2.7
Glutamic acid + alanine	22.7	21.6	21.6	17.3	21.5	21.7
Threonine	5.3	4.9	5.6	5.1	5.0	5.6
Aspartic acid	9.2	6.1	4.7	6.7	4.6	5.7
Serine	5.0	4.4	5.1	3.9	4.5	4.5
Glycine	9.5	7.2	7.4	14.2	7.7	7.5
Arginine+NH_3	22.7	22.5	22.0	22.3	—	—
Lysine	5.8	7.0	5.7	2.8	—	

TABLE III

Relative Molar Concentration of Amino Acids

(Dowex 50 chromatographic analyses of *Mycobacterium tuberculosis* H37Rv cells)

Amino acids	Chromatogram No. 14	15	16	17	18	24	25	27	32	35
Leucine	7.3	7.3	7.4	6.5	10.7	8.9	11.0	8.1	8.2	7.9
Isoleucine	4.5	4.3	4.5	2.2	3.9	7.3	3.8	4.0	3.9	3.3
Phenylalanine	2.4	2.2	2.2	2.5	2.6	1.2	1.9	2.4	2.8	3.0
Valine	7.0	6.6	6.6	6.0	5.4	6.2	6.4	7.1	6.0	5.0
Methionine	3.2	2.5	2.6	1.3	3.8	2.9	2.4	2.7	1.0	2.5
Tyrosine	1.5	0.7	1.6	1.5	1.5	0.9	2.5	1.5	1.5	1.8
Proline	3.2	—	3.1	2.5	2.3	3.0	2.8	1.9	2.7	2.8
Glutamic acid	9.7	—	10.2	9.7	9.3	9.5	11.5	9.5	9.7	10.4
Alanine	12.2	—	11.4	13.5	11.6	11.9	12.2	14.3	15.1	11.5
Threonine	6.5	—	5.1	5.5	6.9	4.8	4.7	5.5	4.9	5.5
Aspartic acid	7.4	7.8	7.0	6.7	7.9	7.7	6.7	8.3	6.1	7.6
Serine	4.7	—	6.5	6.8	5.0	4.3	4.5	4.8	4.0	4.5
Glycine	7.8	—	6.1	6.2	6.6	7.4	7.2	7.5	6.7	7.5

TABLE IV

Relative Molar Concentration of Amino Acids

(Dowex 50 chromatographic analyses of *Mycobacterium tuberculosis* H37RvSR cells)

Amino acids	Chromatogram No. 19	20	21	22	23	26	38
Leucine	8.3	10.6	8.3	7.5	7.1	12.9	8.2
Isoleucine	3.7	3.7	5.4	2.2	3.8	3.0	4.9
Phenylalanine	2.4	2.2	2.6	1.7	2.2	2.9	2.6
Valine	6.3	6.0	4.4	4.5	5.8	6.3	5.6
Methionine	2.3	6.3	2.0	2.0	2.1	1.9	2.4
Tyrosine	1.6	1.5	1.1	1.7	1.5	1.1	1.7
Proline	3.3	2.3	3.2	3.2	2.3	1.6	2.3
Glutamic acid	11.8	7.4	12.5	11.1	10.4	9.5	11.1
Alanine	13.3	11.1	12.3	12.5	11.3	13.2	13.1
Threonine	5.1	6.5	4.7	5.2	—	6.1	5.5
Aspartic acid	7.1	6.9	6.7	—	—	6.6	7.0
Serine	4.4	6.4	4.5	4.8	5.0	4.7	5.2
Glycine	7.7	6.5	8.1	8.4	8.1	7.4	7.8

TABLE V

Relative Molar Concentration of Basic Amino Acids

(Dowex 50 chromatographic analyses of *M. tuberculosis* H37Rv and H37RvSR cells)

Amino acids	H37Rv Chromatogram No. 28	29	34	37	H37RvSR Chromatogram No. 30	31	33	36
Arginine	9.1	9.0	9.5	9.5	10.2	8.0	8.9	10.4
Lysine	3.9	3.7	3.4	3.9	3.8	5.6	4.1	3.0
Histidine	2.2	2.8	2.0	2.5	2.0	2.5	2.9	2.7

DISCUSSION

Of the non-basic amino acids, glutamic acid and alanine were present in the largest proportions in the defatted tubercle bacilli (Tables II–IV) and in the tuberculin protein (Table I). Leucine was also present in relatively high amount, while tyrosine and phenylalanine were present in relatively low amounts. Cystine was not detected in most of the chromatograms. This may be due partly to its low concentration (*7*, *8*), and in the Dowex chromatograms to the criticalness of the pH of the buffer for its separation (*12*). Of the basic amino acids, arginine was present in the largest amount (Tables I, II and V). In the starch column chromatograms part of the arginine peak is due to ammonia which was not taken into consideration in calculating the results. The lower values obtained on the Dowex chromatograms incated that a fairly large proportion of this peak was due to ammonia. (These studies were done on tubercle bacilli exposed to radioactive substrates in the presence and absence of streptomycin, and the displacement of the radioactivity peak also indicated the presence of a large amount of ammonia in the arginine peak in the starch chromatograms. Detailed radioactivity data on these studies will be presented in a later publication).

Some variations in the relative molar proportions of the amino acids were noted from experiment to experiment, but no significant difference between the sensitive and the resistant organisms was evident. Also, no consistently outstanding difference was noted between the values for the tuberculin protein and the bacterial cell protein. In the tables occasional figures are present which are widely divergent from the rest of the values. These are probably due to experimental errors, and do not represent actual differences. Some of the errors and omissions in the tables were due to unavoidable mechanical failure of the fraction collector with consequent disruption of the orderly collection of the effluent.

The values obtained for the tuberculin protein are in fairly good agreement with those obtained by Seibert and her coworkers (*14*) on proteins A and C of the tuberculin. Most of the differnces are within experimental error, or may be attributable to differences in experimental methods and to the fact that Seibert's work was done with purified single protein fractions, while our work was with a mixture of tuberculin proteins as obtained by ammonium sulfate precipitation. It has been observed by Seibert that there were differences in the nitrogen distribution of tuberculins prepared by different methods and from different sources (*15*).

The chromatographic ninhydrin analysis curves of the hydrolysates of defatted *Mycobacterium tuberculosis* H37Rv and H37Ra published by Stein and Moore (*1*) indicated the presence of relatively high proportions of glutamic acid-alanine and leucine, which is in keeping with our results. They were apparently also unable to detect cystine, for no peak representing this amino acid was shown in either of their analysis curves. The results of the analysis of the cell protein of a human tubercle bacillus by Eguchi (*2*) are somewhat at variance with our results. The greatest discrepancies are in the values for methionine and for aspartic acid. His value for methionine is very much larger than ours, while his value for aspartic acid is much smaller. These differences may be due to differences in experimental conditions and the strain of organisms used. Eguchi did not identify his strain, other than that it was a human strain.

An unidentified ninhydrin reacting substance X noted by Seibert in her work with the tuberculin proteins was also found in our chromatograms of both the tuberculin and the cell proteins. It just preceded the appearance of the first known amino acid peak in both the starch and the Dowex 50 chromatograms. Also, in the Dowex chromatograms, another unknown ninhydrin reacting substance appears to be present in the vicinity of the methionine peak, usually overlapping at the end. This substance had an Rf value of 0.66 on a filter paper chromatogram developed with phenol, while the peak presumed to be methionine had a value of 0.80, which is very close to the values established by others (*16*). The presence of this substance somewhat hindered the accurate dermination of methionine, and may in part explain the variation noted in the concentration of methionine from experiment to experiment.

In view of our observation of no difference between the two strains, it may be of interest, also, to mention that Peterson and Pope (*17*) reported that leucine, isoleucine, and valine were found in greater amounts, while histidine and tryptophan were slightly decreased in the Proskauer and Beck culture filtrates of *M. tuberculosis* H37Rv streptomycin resistant strain as compared to the sensitive strain. They used microbiological assay methods for their studies.

SUMMARY

Amino acid analyses of the tuberculin protein and of defatted *M. tuberculosis* H37Rv streptomycin sensitive and streptomycin resistant variants were done by the starch and Dowex 50 chromatographic ninhydrin methods of Moore and Stein.

Glutamic acid, alanine, and leucine were present in relatively high amounts. Tyrosine and phenylalanine were present in relatively low amounts. Among the basic amino acids arginine was present in the largest amount. No significant difference in the amino acid compositions of the sensitive and the resistant strains was noted. Also, no

outstanding difference was observed between the tuberculin protein and the bacterial cell protein.

I wish to thank Dr. C. Richard Smith for his indispensible guidance and advice and for the use of the facilities of the Laboratory of Barlow Sanatorium. I also wish to acknowledge my indebtedness to Mr. Cornelius Y. Chiamori and Mr. Elmer Rice of the University of Southern California, School of Medicine, Department of Biochemistry, and to Mrs. Annie Fry and Mrs. Annie Pearl Lockett for their invaluable assistance.

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CHANGES IN CHEMICAL CONSTITUENTS DURING THE GERMINATION STAGE OF A BEAN, VIGNA SESQUIPEDALIS*

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In the germination stage**, the growth*** of anabolic tissues of bean seed embryo is brought about, by and large, at the expense of nutrients supplied from cotyledon, the reserve organ, of catabolic nature. It has been suggested in the preceding papers (*1*, *2*) that the anabolic or catabolic nature of a given tissue of the embryo is presumably determined by the energy-rich phosphate level in the tissue concerned.

As a matter of course, in the anabolic tissues of seed embryo the distribution of growth rate in respect to time and region is to be seen. Thus in the seedling of *Vigna sesquipedalis*, a predominant growth occurs in the young stem, in which, in turn, the growth of hypocotyl always precedes that of epicotyl (the so-called epigeal germination). In correspondence with such a pattern of growth, the presence of spatial and/or chronological differentiations would naturally be expected in the chemistry of growth process in respective organs of the seed embryo in question.

The study to be reported below is concerned with the changes in various tissue constituents during the germination stage of the embryonic

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** The germination stage is tentatively defined as the heterotrophic stage in which the growth of seedling plant depends entirely on the storage materials of cotyledon. Under the conditions used (sand-culture at 30° in the dark), the time course of growth of sprout of *Vigna sesquipedalis* follows a sigmoid curve with cessation of growth on about the 6th day after the seed sowing, and thereafter the decrease in dry weight of sprout, probably due to autolytic degradation, sets in. In this series of investigations, therefore, this first 6-day period after the sowing was regarded as the germination stage (*1*, *2*).

*** Unless otherwise stated, the word growth signifies increase in dry weight.

organs of *Vigna sesquipedalis*. The results of this study make it clear that the remarkable growth of hypocotyl involves two phases in succession, *i.e.*, first, the growth accompanied with an increase in protoplasmic protein, and secondly the growth without the occurrence of protoplasmic protein deposition. The shift from the first phase to the second takes place at about the middle of the germination stage under the conditions used. This is in striking contrast with the other anabolic organs, plumules and radicles, whose growth is constantly accompanied with the protein accumulation throughout the germination stage. Moreover, a possible role of pentose nucleic acid as a determinant of the growth pattern accompanied with the protein accumulation is pointed out, and the epigeal germination is interpreted in terms of acropetal migration of this growth pattern along the longitudinal axis of the sprout from hypocotyl into epicotyl.

MATERIALS AND METHODS

Bean Seeds—Seeds of *Vigna sesquipedalis* stored in a desiccator for several months after harvest were used. General method for germinating the seeds and preparing excised organs from the seedling plants of various ages has been described previously (*2*). In the present study as the 0-day-old materials dry seeds presoaked in water at 30° for 5 hrs. were employed.

Dry Weight—An appropriate number (from 10 to 100, according to the size of organs examined) of excised organs were dried at 100±5° until constant weights were attained. The dry matters were cooled in a desiccator over silica gel before weighing.

Water-soluble and Water-insoluble Fractions—Tissue constituents were first divided into two fractions with regard to water-solubility. In each case 150 to 250 excised organs were homogenized in a Waring blendor or a glass homogenizer. The homogenate was then placed in a 3G-3 glass filter, and extracted several times with cold water. The residue was boiled for 30 min. with an appropriate amount of water, then cooled and filtered through the same glassfilter used above. The matter remaining on the filter was considered as the water-insoluble fraction, and the combined water extracts as the water-soluble fraction.

Analysis of the Water-soluble Fraction—The water-soluble fractions were analyzed for total sugars, polysaccharides, and soluble nitrogen. Pentose nucleic acid (PNA) and protein were estimated for whole tissue homogenates without previous fractionation in respect to their water-solubility. PNA- and protein contents of water-insoluble fractions of respective organs have been preliminarily confirmed to be negligibly small.

Total Sugars—The water-soluble fraction was extracted with boiling 85 per cent ethyl alcohol. After being freed from alcohol as sufficiently as possible, each extract was hydrolyzed with 1 per cent hydrochloric acid for an hour in an autoclave at 15 lbs. The hydrolysate was neutralized with sodium hydroxide, and made clear with the addition of neutral lead acetate, deleaded with sodium oxalate, centrifuged, and the reducing power of the supernatant solution was titrated by Somogyi's method (*4*). The content of total sugars in the fraction was expressed in terms of equivalent amounts of glucose.

Polysaccharides—The alcohol-insoluble matter obtained in the above procedure of sugar estimation was hydrolyzed, and the reducing power was titrated by the same method described above. Polysaccharide content of the soluble fraction was thus determined in terms of equivalent reducing sugar.

Soluble Nitrogen—The water-soluble fraction was treated with 10 per cent trichloroacetic acid. Total nitrogen content of the supernatant was estimated by Yagi's modification (*5*) of Levy-Palmer's method (*6*).

PNA—The organs separated from 5 to 25 seedling plants were homogenized thoroughly at 0°. After the removal of the acid-soluble and lipid fractions by Schneider's method (*7*), the PNA fraction was obtained according principally to the method of Ogur and Rosen (*8*). An aliquot of the fraction was subjected to digestion in a zinc chloride bath at 150° for 1.5 hrs. To complete the digestion, it was reheated for 30 min. after 3 drops of 30 per cent hydrogen peroxide were added. The clear digest was estimated for phosphorus (considered as PNA phosphorus) by the method of Fiske-Subbarow (*9*). It was experienced that, probably because of contamination with pentosans and polyuronides, the orcinol test for the present materials always gave much higher value of phosphorus in comparison with the direct determination of phosphorus (*cf. 8*).

Protein—The final residue in the above procedure of PNA extraction was treated with 1 N perchloric acid for 20 min. at 70° to remove desoxypentose nucleic acid, and the total nitrogen content of the residue obtained by the modified method of Levy and Palmer was considered to be the protein nitrogen.

Analysis of the Water-insoluble Fraction—The water-insoluble fraction of hypocotyl was analyzed for fats and waxes, pectic substances, proteins, hemicelluloses, and cellulose by the same serial extraction procedure as cited in Christiansen and Thimann (*10*).

Each estimation was carried out at least in triplicate, and representative results are illustrated in the text.

RESULTS

Growth—Growth of each organ was estimated (Fig. 1). The most prominent growth of young stem and the epigeal nature of the germination were clearly demonstrated. Thus, the weight of young stem (inclusive of hypocotyl and epicotyl) finally amounted as much as about 80 per cent of the total weight of the whole sprout attained at the end of the germination stage. The growth of epicotyl was entirely preceded by the growth of hypocotyl, and hardly proceeded until the middle of the germination stage, when epicotyl began to grow with a remarkable growth

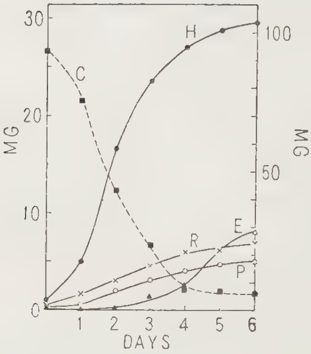

FIG. 1. Changes in dry weight of embryonic organs of *Vigna sesquipedalis* during the germination stage.

P: a pair of plumules E: epicotyl
H: hypocotyl R: radicle
C: a pair of cotyledons

Scales on the right-hand ordinate, for C; the left-hand, for P,E,H, and R.

Values per one individual are plotted.

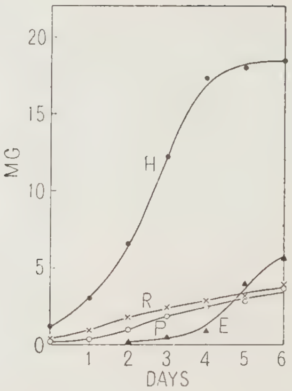

FIG. 2. Changes in water-soluble matter content of embyonic organs of *Vigna sesquipedalis* during the germination stage.

See text of Fig. 1 for symbols used. Values per one individual are plotted.

rate that was comparable to the maximum growth rate shown by the hypocotyl in the earlier germination stage.

Water-soluble Fraction—Change in amount of the water-soluble fraction of each organ in the germination stage was investigated (Fig. 2). This fraction may involve the protoplasmic as well as storage materials. As for cotyledon, since negligibly small amount of water-insoluble material was obtained by the present method, the total dry matter (cf. Fig. 1) was provisionally considered as being wholly consisted of the soluble fraction. In fact, ill-developed septa were seen microscopically to separate bulk of cotyledon cells from each other.

In general, regardless of the kind of organ, the amounts of the water-soluble matters were found to change almost always keeping pace with respective growth curves illustrated in Fig. 1.

Soluble Sugars—Fig. 3 shows a typical result of the soluble sugar estimations made on each organ excised from the seedling of various ages. It is seen that the sugar content of each organ changes nearly in parallel to the extent of growth of respective organs except that considerable decrease in the sugar content of hypocotyl takes place in the later germination stage, and, moreover, it is worthy of note that only a relatively small amount of sugars is present in cotyledon. This small sugar content of cotyledon may naturally be due to rapid export of sugars into other anabolic tissues. As to the form of migrating sugars, no information is as yet available for the present material. In etiolated seedlings, it has been reported (*11*) that sugar in the form of saccharose is exported from cotyledons to and is split in the anabolic tissues of the sprouts. The evident decrease in sugar content of hypocotyl will at least partly relate to the sugar transport from hypocotyl to epicotyl and/or the conversion of sugars into cell-wall polysaccharides in hypocotyl itself. It is shown in the figure that in epicotyl a vigorous sugar accumulation takes place in the later germination stage when the sugar supply of cotyledon is practically exhausted. And the accumulation of cellulose in hypocotyl is, as will be indicated later, stimulated with the age of the tissue. According to Christiansen and Thimann (*10*) the elongation of etiolated pea stem section occurs with the simultaneous disappearance of a considerable amounts of sugars. But only a small part of it is to be deposited as cell-wall polysaccharides, perhaps being largely consumed in oxidative metabolism and/or by transformation to other types of cell constituents than cell-wall polysaccharides.

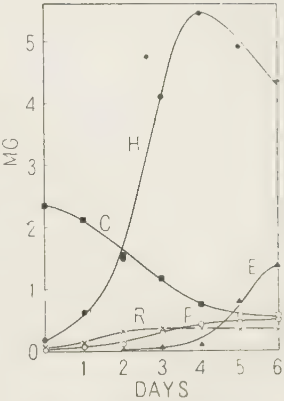

FIG. 3. Changes in soluble sugar content (expressed as equivalent glucose) of embryonic organs of *Vigna sesquipedalis* during the germination stage.

See text of Fig. 1 for symbols used. Values per one individual are plotted.

Polysaccharides—It may be considered that polysaccharides estimated by the present method will be mainly composed of starch. In a separate experiment using the iodine reagent no starch was detectable in the water-insoluble fraction of each organ, whereas with the water-soluble fractions a strong positive reaction was always observed.

Curves which illustrate the time courses of changes in the polysaccharide contents of respective organs (Fig. 4) are also shown to be nearly similar to those of growth, except in the case of plumule. In spite of its rather moderate growth, plumule shows the most remarkable polysaccharide (starch) accumulation. Since starch as such, without previous degradations, would not be transported into plumule, this accumulation would imply a vigorous production of starch in the tissue in question. It would be deduced therefrom that plumule, the predominant region of photosynthesis the apparatus of which is known to be elaborated only under the illuminated conditions, would be provided inherently with most efficient phosphorylasic activity, the finishing step in the photosynthetic starch formation. It is also noticeable that the young stem including both epi- and hypocotyls shows relatively weak activity of polysaccharide deposition in comparison with the extent of growth of the tissue concerned.

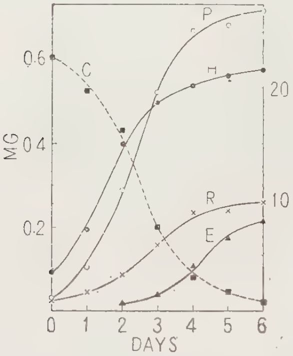

FIG. 4. Changes in soluble polysaccharide content (expressed as equivalent glucose) of embryonic organs of *Vigna sesquipedalis* during the germination stage.

See text of Fig. 1 for symbols used. Scales on the right-hand ordinate, for C; the left-hand, for P, E, H, and R. Values per one individual are plotted.

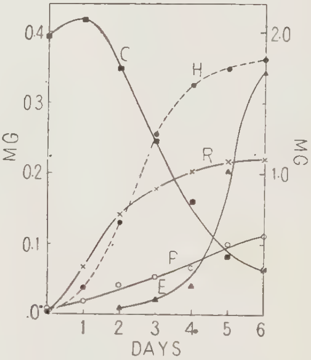

FIG. 5. Changes in soluble nitrogen content of embryonic organs of *Vigna sesquipedalis* during the germination stage.

See text of Fig. 1 for symbols used. Scales on the right-hand ordinate, for H; the left-hand, for P, E, R, and C. Values per one individual are plotted.

Soluble Nitrogen—Change in content of soluble nitrogen of each organ in the germination stage was investigated (Fig. 5). Here again curves roughly similar to those of growth, with an exception of the case of cotyledon, were obtained. Though the dry weight of cotyledon starts a monotonous decline as soon as the seed begins to germinate, there is a slight but definite peak in the soluble nitrogen content on the 2nd day of cultivation. This would relate to the known fact that the protein hydrolysis in cotyledon generally reaches a level of maximum intensity within a day or two after the seed begins to take up water (*12*).

The quantitative ratio, soluble nitrogen vs. protein nitrogen, differs considerably from organ to organ. In the accompanying table, maximum levels of both forms of nitrogen which are attained nearly concurrently in respective tissues are described together. The table indicates that in the anabolic tissues, except plumule, the relation between these two forms of nitrogen is clearly directed toward decomposition. It is of interest that not only plumule but also cotyledon have their respective nitrogen preferentially as protein nitrogen. This fact would relate to the highly synthetic capacity with which the *leaf* organs in general are expected to be provided. It goes without saying that high synthetic activity of cotyledon is detected in its anabolic period when the tissue itself and large amount of storage nutrients were produced in cotyledon on the ovule placenta.

TABLE I

Quantitative Relationship between the Maximum Contents of Soluble Nitrogen and Protein Nitrogen in Embryonic Organs of Vigna sesquipedalis in the Germination Stage

Organ	Plumule	Epicotyl	Hypocotyl	Radicle	Cotyledon
Soluble nitrogen* (*mg.*)	0.11	0.34	1.8	0.22	0.4
Protein nitrogen** (*mg.*)	0.25	0.1	0.3	0.15	2.3
$\frac{\text{Soluble nitrogen}}{\text{Protein nitrogen}}$	0.44	3.4	6.0	1.47	0.17

* Derived from the data illustrated in Fig. 5.
** Derived from the data illustrated in Fig. 6.

PNA and Proteins—Representative results of PNA phosphorus and protein nitrogen estimations are shown in Figs. 6 and 7, respectively. In hypocotyl, the contents of both PNA phosphorus and protein nitrogen increase *pari passu* in the earlier half of the germination stage. However, subsequent behaviours of these two metabolites differ widely from each other, *i.e.*, protein nitrogen remains unchanged over the remainder of the germination stage, whereas PNA phosphorus begins to decline gradually. This is in distinct contrast with the continuous rise in the growth curve of the tissue in question. In other organs, inclusive of cotyledon, both PNA phosphorus and protein nitrogen change almost in parallel with the dry weights of respective organs.

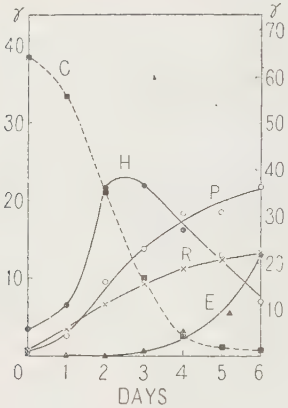

FIG. 6. Changes in pentose nucleic acid phosphorus content of embryonic organs of *Vigna sesquipedalis* during the germination stage.

See text of Fig. 1 for symbols used. Scales on the right-hand ordinate, for C; the left-hand, for P, E, H, and R. Values per one individual are plotted.

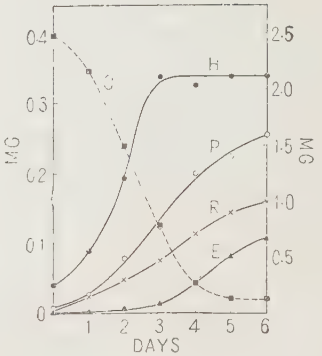

FIG. 7. Changes in protein nitrogen content of embryonic organs of *Vigna sesquipedalis* during the course of the germination stage.

See text of Fig. 1 for symbols used. Scales on the right-hand ordinate, for C; the left-hand, for P, E, H, and R. Values per one individual are plotted.

Unexpected was the fact that a remarkable amount of PNA phosphorus was found to be present in cotyledon at the initial stage of germination. Possible presence of reserve phosphorus such as phytic acid may not be the cause of this high phosphorus value obtained for the PNA fraction, since in the present fractionation procedure both free and bound forms (*cf. 13*) of the acid would be removed together with the acid-soluble and phosphorotein fractions (*cf. 8*). Moreover, a separate experiment conducted by the present authors has shown that a large part of this PNA phosphorus is readily diminished by the action of ribonuclease added.

Water-insoluble Fraction—The time course of change in the amount of the water-insoluble fraction was investigated for each organ (Fig. 8). It may not be unsafe to regard the fraction as being mainly consisted of the cell-wall portion of the cell materials (*cf. 10*). For cotyledon, as stated earlier, little water-insoluble matter was obtained.

Here also the curves are generally of similar nature to those of growth. This would imply that the development of cell-wall is necessarily involved in the embryonic growth of every anabolic tissues in question.

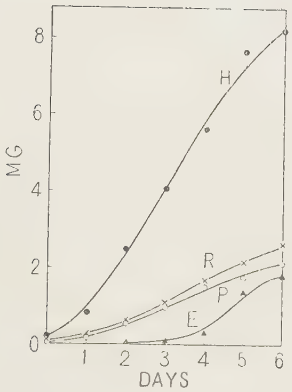

Fig. 8. Changes in water-insoluble matter content of embryonic organs of *Vigna sesquipedalis* during the germination stage.

See text of Fig. 1 for symbols used. Values per one individual are plotted.

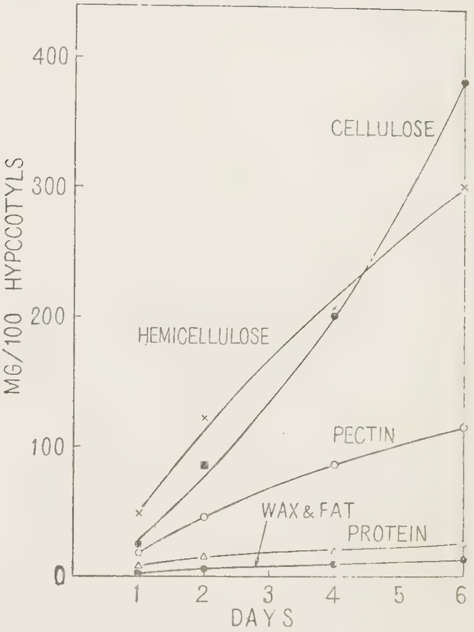

Fig. 9. Changes in cell-wall constituents of hypocotyl of *Vigna sesquipedalis* during the germination stage.

See text of Fig. 1 for symbols used. Values per 100 hypocotyls are plotted.

Cell-wall Constituents of Hypocotyl—To analyze in some detail the cell-wall formation in hypocotyl, quantitative changes in principal constituents of the hypocotyl cell-wall during the germination stage were investigated (Fig. 9). The figure clearly indicates that hemicelluloses and pectic substances are deposited nearly linearly but with slightly decreasing rate, while the amount of cellulose increases in an exponential way throughout the germination stage. Minor constituents such as proteins, fats and waxes were also found to grow in amount linearly.

DISCUSSION

The fact that the whole germination stage is apparently devoted preferentially to the production of the young stem may be of teleological meaning, that is to say, the etiolated sprout can lose no time in stretching its plumules out of the subterranean darkness to receive the sun light since the initiation of autotrophic life by photosynthesis is of urgent necessity in view of the progressive consumption of reserve nutrients.

From the present analyses a striking characteristic of the hypocotyl growth would be deduced, i.e., in hypocotyl, the growth accompanied with protoplasmic protein (PP) deposition is substituted by that accompanied with no PP deposition at about the middle of the germination stage. The cell-wall materials (CW), above all cellulose, however, do not cease to increase in amount throughout the germination stage. It would be convenient to designate provisionally the first mode of growth as the PP-CW growth and the second as the CW growth.* The growth of embryonic organs other than hypocotyl during whole germination stage should then be classified into the PP-CW growth, since evidently it is accompanied with simultaneous accumulation of both PP and CW.

It is increasingly established that PNA would be involved in the protein formation (*14*, *15*). In agreement with this view, every PP-CW growth, but not the CW growth, is shown to be necessarily associated with the concomitant accumulation of PNA. Furthermore, the present results, especially those obtained for hypocotyl, together with those reported by other authors (*e.g.*, *cf. 16*), indicate that neither the mere presence nor disappearance, but the very accumulation of PNA would be in an intimate correlation with the accumulation of protein. Furthermore, it is noted that in every anabolic tissues studied an approximately constant quantitative relationship between maximum accumulation of both protein nitrogen and PNA phosphorus (about 15:1) is found. This appears to imply some direct coupling between the accumulation mechanims of these two metabolites in the present embryonic tissues.

The growth of epicotyl that proceeds in the later half of the germination stage has been revealed to be essentially, relating not only to the growth rate but also to the constituent accumulation, but a repetition of the PP-CW growth of hypocotyl that appears in the earlier half of the germination stage.

It would thus be possible to picture the epigeal germinantion in *Vigna sesquipedalis* in terms of an ascent of growth pattern along the stem axis with the advancement of germination, that is to say, the PP-CW growth would ascend hypocotyl up into epicotyl, leaving the CW growth element alone behind. Relevant to this, attention should be paid to the behaviour of PNA that, as described above, would play an indispensable role in the protein formation, and, therefore, in the PP-CW growth. As Fig. 6 shows, in the later half of the germination stage concurrent accumulation and diminishment of PNA take place in epicotyl and hypocotyl, respectively. The PNA reserve in cotyledon seems to be nearly exhausted by that time, hence presumably the PNA deposition in epicotyl will be performed under the PNA supply from hypocotyl. Hence, it would be presumed that PNA, as such and or after being subjected to enzymic decomposition, will be transported upward from hypocotyl into epicotyl where it may influence the protein accumulation in the sense mentioned above. It has been discussed earlier in the description of the results that sugars would be transported upwards in the stem axis, too. A general acropetal migration of metabolites is thus expected to be involved in the epigeal type of germination. If so, is there to be seen a basipetal migration of PNA and other metabolites from epicotyl into hypocotyl in the case of hypogeal legume plants, in which, contrary to epigeal ones, the hypocotyl growth is preceded by the epicotyl one?

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* To avoid needless confusions, the words "division growth" and "elongation growth" which were used in the previous papers (*1*, *3*) in place of the PP-CW growth and the CW growth, respectively, are abandoned.

On the other hand, the CW element that is commonly contained both in the CW and PP-CW growth patterns of the young stem must naturally have to do directly with the elongation growth. Here auxin does interfere. The authors, however, do not try to go further on this problem; an interesting article of Gates (*17*) should be consulted instead.

It is known (*14*, *15*) that a large amount of PNA is present in a wide variety of developing tissues, but, excepting protein forming ones, it subsequently diminishes along with the completion of tissue development. Hence, it sounds rather astonishing that the fully mature cotyledon, which has to be lacking in any ability of protein synthesis, does contain such an abundant amount of PNA. In view of the storage nature of most tissue constituents of the fully developed cotyledon, it will be quite plausible to consider the cotyledonous PNA as a sort of storage, which might be transported into the anabolic tissues and there evoke protein production. The detailed investigation on the fate of the storage PNA in cotyledon is now in progress.

SUMMARY

1. The growth of each embryonic organ, *i.e.*, plumule, epicotyl, hypocotyl, radicle, and cotyledon of *Vigna sesquipedalis* was followed

daily during the course of the germination stage, by determining increase in dry weight and water-soluble and water-insoluble fractions. The water-soluble fraction of each organ was further analyzed in respect to sugars, polysaccharides, nitrogen, pentose nucleic acid, and proteins. The water-insoluble fraction of hypocotyls was estimated for fats and waxes, pectic substances, proteins, hemicelluloses, and cellulose.

2. Growth curves of sigmoid shape were obtained for each anabolic organ, whereas cotyledons lost their weight in an exponential way. Growth of epicotyl did not proceed practically until the middle of the germination stage, when it was enhanced to a considerable rate (epigeal germination).

3. With plumules, epicotyls, and radicles, a general trend that tissue constituents examined change in parallel to the extent of growth of respective organs was observed. In hypocotyl, however, there occurred an abrupt ceasing of protein accumulation in the water-soluble fraction and distinct decrease in amount of pentose nucleic acid (PNA) as well as sugars in the same fraction at about the middle of the germination stage. The amount of cell-wall materials grew unceasingly thoughout the stage; especially that of cellulose continued its exponential increase.

4. Cotyledons were found to have a large amount of PNA storage, which together with other tissue constituents studied diminished rapidly as the germination proceeded.

5. A possible role of PNA played in the protein accumulation has been discussed and the epigeal type of germination in the present material was interpreted in terms of acropetal migration of metabolites, above all that of PNA, along the stem axis.

Grateful acknowledgement is made to Prof. T. Mori of this institute for his continuing interest throughout the course of this study.

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STUDIES ON CONJUGATION OF S^{35}-SULFATE WITH PHENOLIC COMPOUNDS

I. EXPERIMENTS WITH LIVER SLICES

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The biological conjugation of sulfate with phenols has been studied by several workers. **Dziewiatkowski** (*1*) has demonstrated that S^{35}-labeled inorganic sulfate is incorporated into phenyl sulfate *in vivo*. **DeMeio** *et al.* (*2*) reported that the synthesis of the conjugated sulfate was accomplished in the presence of liver slices as well as cell-free homogenates when phenol and inorganic sulfate were introduced. They established the condensation by determining the disappearance of free phenol in the medium. Recently **Bernstein** and **McGilvery** (*3, 4*) prepared a soluble enzyme from liver homogenate which promotes the combination of aminophenol and inorganic sulfate.

The work reported here deals primarily with the sulfate conjugation of various phenolic substances in the presence of liver slices using radioactive sulfur as a tracer.

EXPERIMENTAL

Liver Slices—Livers obtained from albino rats were cut into slices in an ice cold Krebs' Ringer-phosphate solution (*5*) of which magnesium sulfate was substituted by magnesium chloride so as to exclude sulfate.

Media—The above sulfate-free Ringer-phosphate solution was used as basic medium. Usually 1 volume of 0.1 *N* sodium succinate solution was added to 9 volumes of the basic medium.

Methods—Each vessel contained 0.3 g. of the liver slices and 5 ml. of the succinate-Ringer-phosphate solution, to which 0.05 to 0.1 ml. of a solution of phenolic compounds or other related substances and 0.1 ml. of S^{35}-sulfuric acid solution, carrier-free or with potassium sulfate as carrier, were added. The activity of radioisotope introduced to the vessel was usually 100 to 500 cpm.

The vessel were shaken under oxygen in a thermostat of 37.5°. At the end of incubation 1 ml. of 20 per cent trichloracetic acid was poured into the vessel. The deproteinized filtrate was mixed with 60 micromoles of potassium sulfate as carrier, and the inorganic sulfate was precipitated by excess of barium chloride. To ensure the removal of isotopic inorganic sulfate, 12 micromoles of the carrier sulfate were added again and precipitated as barium sulfate. This was repeated once more.

The supernatant was heated in a boiling water-bath with 2 ml. of 1 *N* HCl for 15 minutes to hydrolyze the organic sulfate. The liberated inorganic sulfate was precipitated along with added carrier, 60 micromoles of potassium sulfate, as before.

The precipitates were washed with water, resuspended in alcohol, transferred quantitatively into counting dishes and dried. No difficulty was encountered to prepare uniform layer of sample by this technique. The radioactivity was measured by means of a Geiger-Müller counter with thin mica window. Because of almost equal sample thickness no correction for the self-absorption was needed.

RESULTS

Conjugation of Sulfate with Phenol—In confirmation of others (1) inorganic sulfate was found to combine with phenol in the presence of liver slices. If carrier-free S^{35}-sulfate was added to the medium the conjugation progressed at a constant rate for 3 hours as shown in Fig. 1.

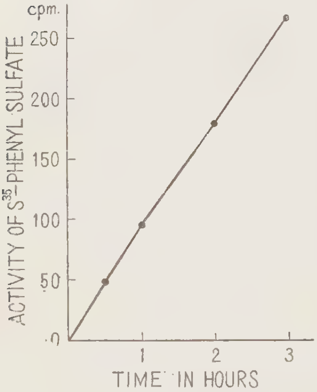

FIG. 1. Conjungation of radioactive sulfate with phenol.

The vessel contained 400 cpm. of carrier-free S^{35}-sulfate, 2 micromoles of phenol and 0.3 g of liver slices.

Liver slices prepared from individual animals showed appreciable differences in the rate of sulfate conjugation, which varied from 21 to 51 per cent, the average being 30 per cent, at the end of 1 hour incubation.

Experiments using various amounts of potassium sulfate and of phenol were carried out. A typical result is given in Table I.

As shown in the table, percentage of combined sulfate increased with higher ratio of phenol to sulfate. The percentage of phenol combined with sulfate was calculated assuming all of the esterified sulfuric acid was in the form of phenyl sulfate. This value increased with higher ratio of added sulfate to phenol. In the extreme case where 1 micromole of phenol and 36 micromoles of sulfate were used the percentage reached 115 per cent. This result may be ascribed to the conjugation of sulfate with some phenol-like substances pre-existing in liver tissue.

TABLE I

Effect of Varying Amount of Sulfate and Phenol in Medium

Sulfate added (*micromoles*)	Phenol added (*micromoles*)		
	1	3	10
36	3 (115)	5 (58)	
12	8 (96)	15 (60)	
3	12 (36)	27 (27)	
Carrier-free	42	47	47

Figures represent percentages of sulfate combined with phenol. Figures in parentheses represent percentages of phenol combined with sulfate.

Liver: 0.6 g.

Identification of Phenyl Sulfate—The precipitation reaction with acriflavin (*6*) was employed for identification of the sulfuric ester produced.

One g. of liver slices was incubated in 10 ml. of succinate-Ringer-phosphate solution with 6 micromoles of phenol and potassium sulfate equivalent to 0.1 mg. of sulfur with activity of 1500 cpm. for 2 hours at 37.5° under oxygen. At the end of incubation the mixture was deproteinized with 2 ml. of 20 per cent trichloracetic acid and the activity of conjugated sulfate in the filtrate was measured (Table II, A).

Fifty mg. (287 micromoles) of phenylsulfate were added to the filtrate and precipitated by acriflavin. The precipitate was washed twice with potassium sulfate solution and twice with water, and its activity was measured (Table II, B).

The compound was then recrystallized from a small amount of alcohol and again the activity was measured (Table II, C).

A control experiment was carried out as above described except that the addition of phenol was omitted, in order to correct the interference by sulfate-binding substances present in liver tissue.

TABLE II

Identification of Phenyl Sulfate

Sample	Activity	Quantity	Specific activity
	cpm.	*micromoles*	*cpm./micromoles*
A	317	290*	1.09
B	76	72	1.06
C	50	47	1.06

* Estimated quantity of carrier plus synthesized phenyl sulfate.

As shown in Table II, the specific activities at the stages A, B and C are in excellent agreement, so it can be deduced that the synthesized ester was identical with phenyl sulfate.

Anaerobiosis and Inhibitors—Table III summarizes observations on the effects of anaerobiosis and of several inhibitors.

For the anaerobic experiment the reaction vessels were flushed with nitrogen gas. The result shows that anaerobiosis inhibited completely the conjugation of phenol and inorganic sulfate. The oxygen pressure in air was insufficient to support the full development of the synthetic reaction, the rate of synthesis being reduced one-half the rate under oxygen.

The effect of oxidation and phosphorylation inhibitors were also conspicuous. 0.002 *M* cyanide or arsenite, or 0.0004 *M* dinitrophenol inhibited the reaction by 95 per cent; azide, fluoride and iodoacetate were somewhat less inhibitory. Unexpectedly, arsenate did not affect the synthesis at the concentration of 0.002 *M*, probably owing to the

TABLE III

Effect of Inhibitors on Sulfate Conjugation

Inhibitor	Approximate final concentration	Inhibition
	M	%
Under air		46–58
Anaerobiosis		100
Cyanide	0.0004	80
Cyanide	0.002	95
Azide	0.002	75
Fluoride	0.002	87
Iodacetate	0.002 (at pH 7.1)	80
Arsenate	0.002	0
Arsenite	0.002	95
p-Nitrophenol	0.0004	75
Pentachlorophenol	0.000075	100
2,4-Dinitrophenol	0.0004	95
Picrate	0.00025	50
Salicylate	0.0004	62
Folic acid	0.004	38
Phlorhidzin	0.004	15
Acriflavin	0.004	0
Cysteine	0.004	0

presence of enough phophate in the medium to compete with arsenate in low concentration.

It was shown that phenol derivatives with acidic radicals such as *p*-nitrophenol, pentachlorophenol, picrate and salicylate were strong inhibitors in considerably lower concentration. On the contrary, folic acid, phlorhidzin, acriflavin or cysteine exhibited only slight or no inhibition.

Experiments with Phenolic Compounds Other than Phenol—About 40

TABLE IV

Sulfate Conjugation of Phenolic Substances Other than Phenol

Substance	Amount of the substance added	Extent of sulfate conjugation	Amount of phenol added in the parallel experiment
	μg.	%	μg.
None		3	200
p-Cresol	300	110	200
m-Aminophenol	300	160–180	200
p-Aminophenol	300	10*	200
o-Nitrophenol	300	66–90	200
m-Nitrophenol	300	63–100	200
p-Nitrophenol	200	13	200
2,4-Dinitrophenol	100	5	100
Salicylaldehyde	160	70	100
Adrenaline	300	20–25	200
Noradrenaline (*dl*)	300	60	200
Resorcinol	100	120	100
Phloroglucinol	100	56	100
α-Naphthol**	300	70	200
β-Naphthol**	300	85	200
8-Hydroxyquinoline	400	90	100
Estrone***	1000	13	100
Estrone benzoate	100	3	200
p-Nitrophenyl sulfate	400	26	100
p-Nitrophenyl phosphate	400	50–70	200
Phenyl sulfate	300	50	200
Phenyl phosphate	200	90	200

The reaction mixtures were incubated for 1 hour under oxygen at 37.5°.

* Darkened rapidly.

** Dissolved in dilute alkali.

*** Dissolved in 0.3 ml. of blood serum.

kinds of substances other than phenol were tested on the ability to combine with sulfate in the presence of liver slices. In Table IV, the extent of sulfate conjugation in each individual experiment is expressed as the percentage of that in the parallel experiment carried out simultaneously with phenol as the reference substance.

As shown in the table, even when no phenolic compound has been added, slight but decided esterification of sulfate was demonstrated. This fact indicates the existence of some substances in liver which combine enzymatically with inorganic sulfate.

Among the substances which combine with inorganic sulfate considerably well, adrenaline and noradrenaline are noticeable in that the sulfate conjugation process may take parts in the inactivation mechanism of adrenaline and noradrenaline *in vivo*. It is well known that estrone is excreted in equine pregnancy urine as sulfate (*7*). The result presented in Table IV shows that estrone may conjugate with sulfate in liver to estrone sulfate which is excreted through kindey. The experiment was carried out with estrone dissolved in blood serum because of its insolubility in saline solution.

Phenols substituted with amino group conjugated readily with inorganic sulfate, except *p*-aminophenol which seems to be oxidized rapidly in the presence of tissue before it combines with sulfate.

There is marked difference among *o*-, *m*- and *p*-nitrophenol. While the former two coupled with inorganic sulfate fairly well, *p*-nitrophenol did only slightly when 200 μg. were used. When the amount of *p*-nitrophenol was raised up to 400 μg. the conjugation did not take place at all. In contrast to *p*-nitrophenol, *p*-nitrophenol phosphate or sulfate was able to take up inorganic sulfate in some extent. The fact may be explained in the following way: *p*-Nitrophenol which inhibits the sulfate conjugation in higher concentration as described above can combine with sulfate if supplied in small portions by gradual hydrolysis of its esters.

Phenols substituted with anionoid radicals such as salicylate, 3,5-dinitrosalicylate, trichlorophenol, pentachlorophenol, 2,4-dinitrophenol, and picrate failed to conjugate with inorganic sulfate. These compounds were shown to act as inhibitors on sulfate conjugation as described above. Tyrosine, phenolphethalein phosphate, bromosulphalein, phenolsulfonphthalein and phlorhidzin also failed to conjugate with inorganic sulfate.

Table V represents the experimental results obtained with aromatic substances which are not phenolic by nature but are known or expected to be converted into phenols *in vivo*. Majority of them were proved to produce sulfuric esters to various extents as shown in the table. Diethyl aniline showed only slight sulfate comjugation and ephedrine did not combine with sulfate.

Organs Other than Liver—The sulfate conjugation of phenol was investigated with kidney, spleen, bone, small intestine, skeletal muscle and skin. In this case the quantity of S^{35} used was of the order of 10,000 cpm. in carrier-free form. Table VI shows that the capacity

TABLE V

Sulfate Conjugation of Aromatic Compounds Other than Phenols

Substance	Amount of the substance added	Extent of sulfate conjugation	Amount of phenol added in the parallel experiment
	μg.	%	μg.
Aniline	200	135	200
Diethylaniline	200	5	200
Nitrobenzene	200	60	200
Benzene	200	37	200
Phenacetin	400	93	200
Veratrol	160	120	100
Indole	100	40	100
Indole	300	100	100
Ephedrine	400	0	100

The reaction mixtures were incubated for 1 hour under oxygen at 37.5°.

TABLE VI

Capacity of Sulfate Conjugation in Different Organs

Organ	Relative extent of sulfate conjugation
Liver	100
Kidney	20
Spleen	2
Bone (femur)	1
Small intestine	2
Skeletal muscle	1
Skin (including subcutaneous tissue)	3

0.3 mg. of organ slices was added with 1 micromole of phenol and carrier free S^{35}-sulfate (10,000 cpm.). The mixture was incubated for 1 hour under oxygen at 37.5°.

of sulfate conjugation of kidney is 20 per cent of that of liver. Other organs exhibted little, if any, sulfate conjugation. Small intestine has been considered to have the capacity of promoting sulfate conjugation comparable to liver (*8*), but in the *in vitro* experiment this could not be confirmed. Whether or not the sulfate conjugation of phenol takes place in tissues other than liver and kidney should be concluded by further studies.

DISCUSSION

The experiments under anaerobic condition and with cyanide suggest that the synthesis of phenyl sulfate in the presence of liver slices is an aerobic process. Furthermore inhibitory effects of iodoacetoate, fluoride, azide, arsenite and 2,4-dinitrophenol provide the evidence to indicate that the energy for the sulfate conjugation is derived from the oxidation of the substrates. That arsenate does not inhibit the synthesis may be explained by the competitive action of phosphate present in the medium in relatively high concentration.

Recently Bernstein and McGilvery (*3*) have succeeded to obtain a soluble enzyme preparation which enables the anaerobic synthesis of *m*-aminophenyl sulfate in the presence of ATP. They (*4*) have demonstrated in later experiments that the preliminary step for the conjugation is an enzymatic activation of inorganic sulfate by ATP. We have attempted to obtain direct evidence for ATP-dependence of the reaction by following the procedures given by Bernstein and McGilvery and will report later.

Comparing the rates of sulfate conjugation of various phenolic compounds, it has been seen that the introduction of cationoid group into the benzene ring increases the tendency to combine with sulfate. This rule is in agreement with what Williams (*6*) has presented by *in vivo* experiments. The anionoid group or halogen reduces the rate of sulfate conjugation.

Several aromatic compounds have been shown to take up inorganic sulfate in the presenge of liver slices. Liver is the site of synthesis of indoxyl sulfate from indole. The same can be concluded from the experiments with benzene, nitrobenzene, aniline, veratrol and phenacetin. Aniline which is believed to be converted largely to *p*-aminophenol takes up the inorganic sulfate more than phenol. Diethylaniline and ephedrine seem to be resistant to biological oxidation in liver. The studies of the sulfate conjugation by means of isotopic sulfate may offer valuable informations about the metabolic pathway of various substances in animal body.

SUMMARY

1. The conjugation of sulfate with various phenolic compounds and related substances in the presence of liver slices was studied by means of S^{35}-sulfate.

2. The percentage of sulfate incorporated into phenyl sulfate was greater, the lower the ratio of sulfate to phenol.

3. Anaerobiosis as well as oxidation and phosphorylation inhibitors such as cyanide, fluoride, azide, iodoacetate and dinitrophenol inhibit the synthesis of phenyl sulfate.

4. If cationoid group is introduced into the benzene ring of phenol the extent of sulfate conjugation increases. On the contrary anionoid group or halogen decreases the sulfate conjugation.

5. Indole, benzene, aniline and other aromatic substances can combine with inorganic sulfate probably after they are oxidized to phenols.

6. Kidney is the only organ other than liver which promotes the synthesis of phenol sulfate in considerable extent.

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